EXHIBIT G

INTERNATIONAL COUNCIL FOR HARMONISATION OF TECHNICAL REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE (ICH)

ICH HARMONISED GUIDELINE

ASSESSMENT AND CONTROL OF DNA REACTIVE (MUTAGENIC) IMPURITIES IN PHARMACEUTICALS TO LIMIT POTENTIAL CARCINOGENIC RISK

M7(R1)

Current *Step 4* version

dated 31 March 2017

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the ICH regions.

M7(R1)
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Current *Step 4* version

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ICH Harmonised Guideline

Having reached *Step 4* of the ICH Process at the ICH Assembly meeting on 31 May 2017, this Guideline is recommended for adoption to the regulatory parties to ICH.

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ASSESSMENT AND CONTROL OF DNA REACTIVE (MUTAGENIC) IMPURITIES IN PHARMACEUTICALS TO LIMIT POTENTIAL CARCINOGENIC RISK

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1. INTRODUCTION

The synthesis of drug substances involves the use of reactive chemicals, reagents, solvents, catalysts, and other processing aids. As a result of chemical synthesis or subsequent degradation, impurities reside in all drug substances and associated drug products. While ICH Q3A(R2): Impurities in New Drug Substances and Q3B(R2): Impurities in New Drug Products (Ref. 1, 2) provides guidance for qualification and control for the majority of the impurities, limited guidance is provided for those impurities that are DNA reactive. The purpose of this guideline is to provide a practical framework that is applicable to the identification, categorization, qualification, and control of these mutagenic impurities to limit potential carcinogenic risk. This guideline is intended to complement ICH Q3A(R2), Q3B(R2) (Note 1), and ICH M3(R2): Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorizations for Pharmaceuticals (Ref. 3).

This guideline emphasizes considerations of both safety and quality risk management in establishing levels of mutagenic impurities that are expected to pose negligible carcinogenic risk. It outlines recommendations for assessment and control of mutagenic impurities that reside or are reasonably expected to reside in final drug substance or product, taking into consideration the intended conditions of human use.

2. SCOPE OF GUIDELINE

This document is intended to provide guidance for new drug substances and new drug products during their clinical development and subsequent applications for marketing. It also applies to post-approval submissions of marketed products, and to new marketing applications for products with a drug substance that is present in a previously approved product, in both cases only where:

- Changes to the drug substance synthesis result in new impurities or increased acceptance criteria for existing impurities;
- Changes in the formulation, composition or manufacturing process result in new degradation products or increased acceptance criteria for existing degradation products;
- Changes in indication or dosing regimen are made which significantly affect the acceptable cancer risk level.

Assessment of the mutagenic potential of impurities as described in this guideline is not intended for the following types of drug substances and drug products: biological/biotechnological, peptide, oligonucleotide, radiopharmaceutical, fermentation products, herbal products, and crude products of animal or plant origin.

This guideline does not apply to drug substances and drug products intended for advanced cancer indications as defined in the scope of ICH S9 (Ref. 4). Additionally, there may be some cases where a drug substance intended for other indications is itself genotoxic at therapeutic concentrations and may be expected to be associated with an increased cancer risk. Exposure to a mutagenic impurity in these cases would not significantly add to the

cancer risk of the drug substance. Therefore, impurities could be controlled at acceptable levels for non-mutagenic impurities.

Assessment of the mutagenic potential of impurities as described in this guideline is not intended for excipients used in existing marketed products, flavoring agents, colorants, and perfumes. Application of this guideline to leachables associated with drug product packaging is not intended, but the safety risk assessment principles outlined in this guideline for limiting potential carcinogenic risk can be used if warranted. The safety risk assessment principles of this guideline can be used if warranted for impurities in excipients that are used for the first time in a drug product and are chemically synthesized.

3. GENERAL PRINCIPLES

The focus of this guideline is on DNA reactive substances that have a potential to directly cause DNA damage when present at low levels leading to mutations and therefore, potentially causing cancer. This type of mutagenic carcinogen is usually detected in a bacterial reverse mutation (mutagenicity) assay. Other types of genotoxicants that are non-mutagenic typically have threshold mechanisms and usually do not pose carcinogenic risk in humans at the level ordinarily present as impurities. Therefore to limit a possible human cancer risk associated with the exposure to potentially mutagenic impurities, the bacterial mutagenicity assay is used to assess the mutagenic potential and the need for controls. Structure-based assessments are useful for predicting bacterial mutagenicity outcomes based upon the established knowledge. There are a variety of approaches to conduct this evaluation including a review of the available literature, and/or computational toxicology assessment.

A Threshold of Toxicological Concern (TTC) concept was developed to define an acceptable intake for any unstudied chemical that poses a negligible risk of carcinogenicity or other toxic effects. The methods upon which the TTC is based are generally considered to be very conservative since they involve a simple linear extrapolation from the dose giving a 50% tumor incidence (TD_{50}) to a 1 in 10^6 incidence, using TD_{50} data for the most sensitive species and most sensitive site of tumor induction. For application of a TTC in the assessment of acceptable limits of mutagenic impurities in drug substances and drug products, a value of 1.5 µg/day corresponding to a theoretical 10^{-5} excess lifetime risk of cancer, can be justified. Some structural groups were identified to be of such high potency that intakes even below the TTC would theoretically be associated with a potential for a significant carcinogenic risk. This group of high potency mutagenic carcinogens referred to as the "cohort of concern", comprises aflatoxin-like-, N-nitroso-, and alkyl-azoxy compounds.

During clinical development, it is expected that control strategies and approaches will be less developed in earlier phases where overall development experience is limited. This guideline bases acceptable intakes for mutagenic impurities on established risk assessment strategies. Acceptable risk during the early development phase is set at a theoretically calculated level of approximately one additional cancer per million. For later stages in development and for marketed products, acceptable increased cancer risk is set at a theoretically calculated level of approximately one in one hundred thousand. These risk levels represent a small theoretical increase in risk when compared to human overall lifetime incidence of developing any type of cancer, which is greater than 1 in 3. It is noted that established cancer risk assessments are based on lifetime exposures. Less-Than-Lifetime (LTL) exposures both during development and marketing can have higher acceptable intakes of impurities and still maintain comparable risk levels. The use of a numerical cancer risk value (1 in 100,000) and its translation into risk-based doses (TTC) is a highly hypothetical concept that should not be regarded as a realistic indication of the actual risk. Nevertheless, the TTC concept provides an estimate of

safe exposures for any mutagenic compound. However, exceeding the TTC is not necessarily associated with an increased cancer risk given the conservative assumptions employed in the derivation of the TTC value. The most likely increase in cancer incidence is actually much less than 1 in 100,000. In addition, in cases where a mutagenic compound is a non-carcinogen in a rodent bioassay, there would be no predicted increase in cancer risk. Based on all the above considerations, any exposure to an impurity that is later identified as a mutagen is not necessarily associated with an increased cancer risk for patients already exposed to the impurity. A risk assessment would determine whether any further actions would be taken.

Where a potential risk has been identified for an impurity, an appropriate control strategy leveraging process understanding and/or analytical controls should be developed to ensure that the mutagenic impurity is at or below the acceptable cancer risk level.

There may be cases when an impurity is also a metabolite of the drug substance. In such cases the risk assessment that addresses mutagenicity of the metabolite can qualify the impurity.

4. CONSIDERATIONS FOR MARKETED PRODUCTS

This guideline is not intended to be applied retrospectively (i.e., to products marketed prior to adoption of this guideline). However, some types of post-approval changes warrant a reassessment of safety relative to mutagenic impurities. This section applies to these post-approval changes for products marketed prior to, or after, the adoption of this guideline. Section 8.5 (Lifecycle Management) contains additional recommendations for products marketed after adoption of this guideline.

4.1 Post-Approval Changes to the Drug Substance Chemistry, Manufacturing, and Controls

Post-approval submissions involving the drug substance chemistry, manufacturing, and controls should include an evaluation of the potential risk impact associated with mutagenic impurities from changes to the route of synthesis, reagents, solvents, or process conditions after the starting material. Specifically, changes should be evaluated to determine if the changes result in any new mutagenic impurities or higher acceptance criteria for existing mutagenic impurities. Reevaluation of impurities not impacted by changes is not recommended. For example, when only a portion of the manufacturing process is changed, the assessment of risk from mutagenic impurities should be limited to whether any new mutagenic impurities result from the change, whether any mutagenic impurities formed during the affected step are increased, and whether any known mutagenic impurities from up-stream steps are increased. Regulatory submissions associated with such changes should describe the assessment as outlined in Section 9.2. Changing the site of manufacture of drug substance, intermediates, or starting materials or changing raw materials supplier will not require a reassessment of mutagenic impurity risk.

When a new drug substance supplier is proposed, evidence that the drug substance produced by this supplier using the same route of synthesis as an existing drug product marketed in the assessor's region is considered to be sufficient evidence of acceptable risk/benefit regarding mutagenic impurities and an assessment per this guideline is not required. If this is not the case, then an assessment per this guideline is expected.

4.2 Post-Approval Changes to the Drug Product Chemistry, Manufacturing, and Controls

Post-approval submissions involving the drug product (e.g., change in composition, manufacturing process, dosage form) should include an evaluation of the potential risk associated with any new mutagenic degradation products or higher acceptance criteria for existing mutagenic degradation products. If appropriate, the regulatory submission would include an updated control strategy. Reevaluation of the drug substance associated with drug products is not recommended or expected provided there are no changes to the drug substance. Changing the site of manufacture of drug product will not require a reassessment of mutagenic impurity risk.

4.3 Changes to the Clinical Use of Marketed Products

Changes to the clinical use of marketed products that can warrant a reevaluation of the mutagenic impurity limits include a significant increase in clinical dose, an increase in duration of use (in particular when a mutagenic impurity was controlled above the lifetime acceptable intake for a previous indication that may no longer be appropriate for the longer treatment duration associated with the new indication), or for a change in indication from a serious or life threatening condition where higher acceptable intakes were justified (Section 7.5) to an indication for a less serious condition where the existing impurity acceptable intakes may no longer be appropriate. Changes to the clinical use of marketed products associated with new routes of administration or expansion into patient populations that include pregnant women and/or pediatrics will not warrant a reevaluation, assuming no increases in daily dose or duration of treatment.

4.4 Other Considerations for Marketed Products

Application of this guideline may be warranted to marketed products if there is specific cause for concern. The existence of impurity structural alerts alone is considered insufficient to trigger follow-up measures, unless it is a structure in the cohort of concern (Section 3). However a specific cause for concern would be new relevant impurity hazard data (classified as Class 1 or 2, Section 6) generated after the overall control strategy and specifications for market authorization were established. This new relevant impurity hazard data should be derived from high-quality scientific studies consistent with relevant regulatory testing guidelines, with data records or reports readily available. Similarly, a newly discovered impurity that is a known Class 1 or Class 2 mutagen that is present in a marketed product could also be a cause for concern. In both of these cases when the applicant becomes aware of this new information, an evaluation per this guideline should be conducted.

5. Drug Substance and Drug Product Impurity Assessment

Actual and potential impurities that are likely to arise during the synthesis and storage of a new drug substance, and during manufacturing and storage of a new drug product should be assessed.

The impurity assessment is a two-stage process:

- Actual impurities that have been identified should be considered for their mutagenic potential.
- An assessment of potential impurities likely to be present in the final drug substance is carried out to determine if further evaluation of their mutagenic potential is required.

The steps as applied to synthetic impurities and degradation products are described in Sections 5.1 and 5.2, respectively.

5.1 Synthetic Impurities

Actual impurities include those observed in the drug substance above the ICH Q3A reporting thresholds. Identification of actual impurities is expected when the levels exceed the identification thresholds outlined by ICH Q3A. It is acknowledged that some impurities below the identification threshold may also have been identified.

Potential impurities in the drug substance can include starting materials, reagents and intermediates in the route of synthesis from the starting material to the drug substance.

The risk of carryover into the drug substance should be assessed for identified impurities that are present in starting materials and intermediates, and impurities that are reasonably expected by-products in the route of synthesis from the starting material to the drug substance. As the risk of carryover may be negligible for some impurities (e.g., those impurities in early synthetic steps of long routes of synthesis), a risk-based justification could be provided for the point in the synthesis after which these types of impurities should be evaluated for mutagenic potential.

For starting materials that are introduced late in the synthesis of the drug substance (and where the synthetic route of the starting material is known) the final steps of the starting material synthesis should be evaluated for potential mutagenic impurities.

Actual impurities where the structures are known and potential impurities as defined above should be evaluated for mutagenic potential as described in Section 6.

5.2 Degradation Products

Actual drug substance degradation products include those observed above the ICH Q3A reporting threshold during storage of the drug substance in the proposed long-term storage conditions and primary and secondary packaging. Actual degradation products in the drug product include those observed above the ICH Q3B reporting threshold during storage of the drug product in the proposed long-term storage conditions and primary and secondary packaging, and also include those impurities that arise during the manufacture of the drug product. Identification of actual degradation products is expected when the levels exceed the identification thresholds outlined by ICH Q3A/Q3B. It is acknowledged that some degradation products below the identification threshold may also have been identified.

Potential degradation products in the drug substance and drug product are those that may be reasonably expected to form during long term storage conditions. Potential degradation products include those that form above the ICH Q3A/B identification threshold during accelerated stability studies (e.g., 40°C/75% relative humidity for 6 months) and confirmatory photo-stability studies as described in ICH Q1B (Ref. 5), but are yet to be confirmed in the drug substance or drug product under long-term storage conditions in the primary packaging.

Knowledge of relevant degradation pathways can be used to help guide decisions on the selection of potential degradation products to be evaluated for mutagenicity e.g., from degradation chemistry principles, relevant stress testing studies, and development stability studies.

Actual and potential degradation products likely to be present in the final drug substance or drug product and where the structure is known should be evaluated for mutagenic potential as described in Section 6.

5.3 Considerations for Clinical Development

It is expected that the impurity assessment described in Sections 5.1 and 5.2 applies to products in clinical development. However, it is acknowledged that the available information is limited. For example, information from long term stability studies and photo-stability studies may not be available during clinical development and thus information on potential degradation products may be limited. Additionally, the thresholds outlined in ICH Q3A/B do not apply to products in clinical development and consequently fewer impurities will be identified.

6. HAZARD ASSESSMENT ELEMENTS

Hazard assessment involves an initial analysis of actual and potential impurities by conducting database and literature searches for carcinogenicity and bacterial mutagenicity data in order to classify them as Class 1, 2, or 5 according to Table 1. If data for such a classification are not available, an assessment of Structure-Activity Relationships (SAR) that focuses on bacterial mutagenicity predictions should be performed. This could lead to a classification into Class 3, 4, or 5.

Table 1: Impurities Classification with Respect to Mutagenic and Carcinogenic Potential and Resulting Control Actions

Class	Definition	Proposed action for control (details in Section 7 and 8)
1	Known mutagenic carcinogens	Control at or below compound-specific acceptable limit
2	Known mutagens with unknown carcinogenic potential (bacterial mutagenicity positive*, no rodent carcinogenicity data)	Control at or below acceptable limits (appropriate TTC)
3	Alerting structure, unrelated to the structure of the drug substance; no mutagenicity data	Control at or below acceptable limits (appropriate TTC) or conduct bacterial mutagenicity assay; If non-mutagenic = Class 5 If mutagenic = Class 2
4	Alerting structure, same alert in drug substance or compounds related to the drug substance (e.g., process intermediates) which have been tested and are non-mutagenic	Treat as non-mutagenic impurity
5	No structural alerts, or alerting structure with sufficient data to demonstrate lack of mutagenicity or carcinogenicity	Treat as non-mutagenic impurity

*Or other relevant positive mutagenicity data indicative of DNA-reactivity related induction of gene mutations (e.g., positive findings in *in vivo* gene mutation studies)

A computational toxicology assessment should be performed using (Q)SAR methodologies that predict the outcome of a bacterial mutagenicity assay (Ref. 6). Two (Q)SAR prediction methodologies that complement each other should be applied. One methodology should be

expert rule-based and the second methodology should be statistical-based. (Q)SAR models utilizing these prediction methodologies should follow the general validation principles set forth by the Organisation for Economic Co-operation and Development (OECD).

The absence of structural alerts from two complementary (Q)SAR methodologies (expert rule-based and statistical) is sufficient to conclude that the impurity is of no mutagenic concern, and no further testing is recommended (Class 5 in Table 1).

If warranted, the outcome of any computer system-based analysis can be reviewed with the use of expert knowledge in order to provide additional supportive evidence on relevance of any positive, negative, conflicting or inconclusive prediction and provide a rationale to support the final conclusion.

To follow up on a relevant structural alert (Class 3 in Table 1), either adequate control measures could be applied or a bacterial mutagenicity assay with the impurity alone can be conducted. An appropriately conducted negative bacterial mutagenicity assay (Note 2) would overrule any structure-based concern, and no further genotoxicity assessments would be recommended (Note 1). These impurities should be considered non-mutagenic (Class 5 in Table 1). A positive bacterial mutagenicity result would warrant further hazard assessment and/or control measures (Class 2 in Table 1). For instance, when levels of the impurity cannot be controlled at an appropriate acceptable limit, it is recommended that the impurity be tested in an *in vivo* gene mutation assay in order to understand the relevance of the bacterial mutagenicity assay result under *in vivo* conditions. The selection of other *in vivo* genotoxicity assays should be scientifically justified based on knowledge of the mechanism of action of the impurity and expected target tissue exposure (Note 3). *In vivo* studies should be designed taking into consideration existing ICH genotoxicity Guidelines. Results in the appropriate *in vivo* assay may support setting compound specific impurity limits.

An impurity with a structural alert that is shared (e.g., same structural alert in the same position and chemical environment) with the drug substance or related compounds can be considered as non-mutagenic (Class 4 in Table 1) if the testing of such material in the bacterial mutagenicity assay was negative.

7. RISK CHARACTERIZATION

As a result of hazard assessment described in Section 6, each impurity will be assigned to one of the five classes in Table 1. For impurities belonging in Classes 1, 2, and 3 the principles of risk characterization used to derive acceptable intakes are described in this section.

7.1 TTC-based Acceptable Intakes

A TTC-based acceptable intake of a mutagenic impurity of 1.5 µg per person per day is considered to be associated with a negligible risk (theoretical excess cancer risk of <1 in 100,000 over a lifetime of exposure) and can in general be used for most pharmaceuticals as a default to derive an acceptable limit for control. This approach would usually be used for mutagenic impurities present in pharmaceuticals for long-term treatment (> 10 years) and where no carcinogenicity data are available (Classes 2 and 3).

7.2 Acceptable Intakes Based on Compound-Specific Risk Assessments

7.2.1 Mutagenic Impurities with Positive Carcinogenicity Data (Class 1 in Table 1)

Compound-specific risk assessments to derive acceptable intakes should be applied instead of the TTC-based acceptable intakes where sufficient carcinogenicity data exist. For a known mutagenic carcinogen, a compound-specific acceptable intake can be calculated based on

carcinogenic potency and linear extrapolation as a default approach. Alternatively, other established risk assessment practices such as those used by international regulatory bodies may be applied either to calculate acceptable intakes or to use already existing values published by regulatory authorities (Note 4).

Compound-specific calculations for acceptable intakes can be applied case-by-case for impurities which are chemically similar to a known carcinogen compound class (class-specific acceptable intakes) provided that a rationale for chemical similarity and supporting data can be demonstrated (Note 5).

7.2.2 Mutagenic Impurities with Evidence for a Practical Threshold

The existence of mechanisms leading to a dose response that is non-linear or has a practical threshold is increasingly recognized, not only for compounds that interact with non-DNA targets but also for DNA-reactive compounds, whose effects may be modulated by, for example, rapid detoxification before coming into contact with DNA, or by effective repair of induced damage. The regulatory approach to such compounds can be based on the identification of a No-Observed Effect Level (NOEL) and use of uncertainty factors (ICH Q3C(R5), Ref. 7) to calculate a Permissible Daily Exposure (PDE) when data are available.

The acceptable intakes derived from compound-specific risk assessments (Section 7.2) can be adjusted for shorter duration of use in the same proportions as defined in the following sections (Section 7.3.1 and 7.3.2) or should be limited to not more than 0.5%, whichever is lower. For example, if the compound specific acceptable intake is 15 µg/day for lifetime exposure, the less than lifetime limits (Table 2) can be increased to a daily intake of 100 µg (> 1-10 years treatment duration), 200 µg (> 1-12 months) or 1200 µg (< 1 month). However, for a drug with a maximum daily dose of, for instance, 100 mg the acceptable daily intake for the < 1 month duration would be limited to 0.5% (500 µg) rather than 1200 µg.

7.3 Acceptable Intakes in Relation to LTL Exposure

Standard risk assessments of known carcinogens assume that cancer risk increases as a function of cumulative dose. Thus, cancer risk of a continuous low dose over a lifetime would be equivalent to the cancer risk associated with an identical cumulative exposure averaged over a shorter duration.

The TTC-based acceptable intake of 1.5 µg/day is considered to be protective for a lifetime of daily exposure. To address LTL exposures to mutagenic impurities in pharmaceuticals, an approach is applied in which the acceptable cumulative lifetime dose (1.5 µg/day x 25,550 days = 38.3 mg) is uniformly distributed over the total number of exposure days during LTL exposure. This would allow higher daily intake of mutagenic impurities than would be the case for lifetime exposure and still maintain comparable risk levels for daily and non-daily treatment regimens. Table 2 is derived from the above concepts and illustrates the acceptable intakes for LTL to lifetime exposures for clinical development and marketing. In the case of intermittent dosing, the acceptable daily intake should be based on the total number of dosing days instead of the time interval over which the doses were administered and that number of dosing days should be related to the relevant duration category in Table 2. For example, a drug administered once per week for 2 years (i.e., 104 dosing days) would have an acceptable intake per dose of 20µg.

Table 2: Acceptable Intakes for an Individual Impurity

Duration of treatment	≤ 1 month	>1 - 12 months	>1 - 10 years	>10 years to lifetime
Daily intake [µg/day]	120	20	10	1.5

7.3.1 Clinical Development

Using this LTL concept, acceptable intakes of mutagenic impurities are recommended for limited treatment periods during clinical development of up to 1 month, 1 to 12 months and more than one year up to completion of Phase 3 clinical trials (Table 2). These adjusted acceptable intake values maintain a 10^{-6} risk level in early clinical development when benefit has not yet been established and a 10^{-5} risk level for later stages in development (Note 6).

An alternative approach to the strict use of an adjusted acceptable intake for any mutagenic impurity could be applied for Phase 1 clinical trials for dosing up to 14 days. For this approach, only impurities that are known mutagenic carcinogens (Class 1) and known mutagens of unknown carcinogenic potential (Class 2), as well as impurities in the cohort of concern chemical class, should be controlled (see Section 8) to acceptable limits as described in Section 7. All other impurities would be treated as non-mutagenic impurities. This includes impurities which contain structural alerts (Class 3), which alone would not trigger action for an assessment for this limited Phase 1 duration.

7.3.2 Marketed Products

The treatment duration categories with acceptable intakes in Table 2 for marketed products are intended to be applied to anticipated exposure durations for the great majority of patients. The proposed intakes along with various scenarios for applying those intakes are described in Table 4, Note 7. In some cases, a subset of the population of patients may extend treatment beyond the marketed drugs categorical upper limit (e.g., treatment exceeding 10 years for an acceptable intake of 10 µg/day, perhaps receiving 15 years of treatment). This would result in a negligible increase (in the example given, a fractional increase to 1.5/100,000) compared to the overall calculated risk for the majority of patients treated for 10 years.

7.4 Acceptable Intakes for Multiple Mutagenic Impurities

The TTC-based acceptable intakes should be applied to each individual impurity. When there are two Class 2 or Class 3 impurities, individual limits apply. When there are three or more Class 2 or Class 3 impurities specified on the drug substance specification, total mutagenic impurities should be limited as described in Table 3 for clinical development and marketed products.

For combination products each active ingredient should be regulated separately.

Table 3: Acceptable Total Daily Intakes for Multiple Impurities

Duration of treatment	≤ 1 month	>1 - 12 months	>1 - 10 years	>10 years to lifetime
Total Daily intake [µg/day]	120	60	30	5

Only specified Class 2 and 3 impurities on the drug substance specification are included in the calculation of the total limit. However, impurities with compound-specific or class-related acceptable intake limits (Class 1) should not be included in the total limits of Class 2 and Class 3 impurities. Also, degradation products which form in the drug product would be controlled individually and a total limit would not be applied.

7.5 Exceptions and Flexibility in Approaches

- Higher acceptable intakes may be justified when human exposure to the impurity will be much greater from other sources e.g., food, or endogenous metabolism (e.g., formaldehyde).
- Case-by-case exceptions to the use of the appropriate acceptable intake can be justified in cases of severe disease, reduced life expectancy, late onset but chronic disease, or with limited therapeutic alternatives.
- Compounds from some structural classes of mutagens can display extremely high carcinogenic potency (cohort of concern), i.e., aflatoxin-like-, N-nitroso-, and alkyl-azoxy structures. If these compounds are found as impurities in pharmaceuticals, acceptable intakes for these high-potency carcinogens would likely be significantly lower than the acceptable intakes defined in this guideline. Although the principles of this guideline can be used, a case-by-case approach using e.g., carcinogenicity data from closely related structures, if available, should usually be developed to justify acceptable intakes for pharmaceutical development and marketed products.

The above risk approaches described in Section 7 are applicable to all routes of administration and no corrections to acceptable intakes are generally warranted. Exceptions to consider may include situations where data justify route-specific concerns that should be evaluated case-by-case. These approaches are also applicable to all patient populations based upon the conservative nature of the risk approaches being applied.

8. CONTROL

A control strategy is a planned set of controls, derived from current product and process understanding that assures process performance and product quality (ICH Q10, Ref. 8). A control strategy can include, but is not limited to, the following:

- Controls on material attributes (including raw materials, starting materials, intermediates, reagents, solvents, primary packaging materials);
- Facility and equipment operating conditions;
- Controls implicit in the design of the manufacturing process;
- In-process controls (including in-process tests and process parameters);
- Controls on drug substance and drug product (e.g., release testing).

When an impurity has been characterized as Classes 1, 2, or 3 in Table 1, it is important to develop a control strategy that assures that the level of this impurity in the drug substance and drug product is below the acceptable limit. A thorough knowledge of the chemistry associated with the drug substance manufacturing process, and of the drug product manufacturing process, along with an understanding of the overall stability of the drug substance and drug product is fundamental to developing the appropriate controls. Developing a strategy to control mutagenic impurities in the drug product is consistent with risk management processes identified in ICH Q9 (Ref. 9). A control strategy that is based on product and process understanding and utilisation of risk management principles will lead to a combination of process design and control and appropriate analytical testing, which can also

provide an opportunity to shift controls upstream and minimize the need for end-product testing.

8.1 Control of Process Related Impurities

There are 4 potential approaches to development of a control strategy for drug substance:

Option 1
Include a test for the impurity in the drug substance specification with an acceptance criterion at or below the acceptable limit using an appropriate analytical procedure.

For an Option 1 control approach, it is possible to apply periodic verification testing per ICH Q6A (Ref. 10). Periodic verification testing is justified when it can be shown that levels of the mutagenic impurity in the drug substance are less than 30% of the acceptable limit for at least 6 consecutive pilot scale or 3 consecutive production scale batches. If this condition is not fulfilled, a routine test in the drug substance specification is recommended. See Section 8.3 for additional considerations.

Option 2
Include a test for the impurity in the specification for a raw material, starting material or intermediate, or as an in-process control, with an acceptance criterion at or below the acceptable limit using an appropriate analytical procedure.

Option 3
Include a test for the impurity in the specification for a raw material, starting material or intermediate, or as an in-process control, with an acceptance criterion above the acceptable limit of the impurity in the drug substance, using an appropriate analytical procedure coupled with demonstrated understanding of fate and purge and associated process controls that assure the level in the drug substance is below the acceptable limit without the need for any additional testing later in the process.

This option can be justified when the level of the impurity in the drug substance will be less than 30% of the acceptable limit by review of data from laboratory scale experiments (spiking experiments are encouraged) and where necessary supported by data from pilot scale or commercial scale batches. See Case Examples 1 and 2. Alternative approaches can be used to justify Option 3.

Option 4
Understand process parameters and impact on residual impurity levels (including fate and purge knowledge) with sufficient confidence that the level of the impurity in the drug substance will be below the acceptable limit such that no analytical testing is recommended for this impurity. (i.e., the impurity does not need to be listed on any specification).

A control strategy that relies on process controls in lieu of analytical testing can be appropriate if the process chemistry and process parameters that impact levels of mutagenic impurities are understood and the risk of an impurity residing in the final drug substance above the acceptable limit is determined to be negligible. In many cases justification of this control approach based on scientific principles alone is sufficient. Elements of a scientific risk assessment can be used to justify an option 4 approach. The risk assessment can be based on physicochemical properties and process factors that influence the fate and purge of an impurity including chemical reactivity, solubility, volatility, ionizability and any physical process steps designed to remove impurities. The result of this risk assessment might be shown as an estimated purge factor for clearance of the impurity by the process (Ref. 11).

Option 4 is especially useful for those impurities that are inherently unstable (e.g., thionyl chloride that reacts rapidly and completely with water) or for those impurities that are introduced early in the synthesis and are effectively purged.

In some cases an Option 4 approach can be appropriate when the impurity is known to form, or is introduced late in the synthesis, however process-specific data should then be provided to justify this approach.

8.2 Considerations for Control Approaches

For Option 4 approaches where justification based on scientific principles alone is not considered sufficient, as well as for Option 3 approaches, analytical data to support the control approach is expected. This could include as appropriate information on the structural changes to the impurity caused by downstream chemistry ("fate"), analytical data on pilot scale batches, and in some cases, laboratory scale studies with intentional addition of the impurity ("spiking studies"). In these cases, it is important to demonstrate that the fate/purge argument for the impurity is robust and will consistently assure a negligible probability of an impurity residing in the final drug substance above the acceptable limit. Where the purge factor is based on developmental data, it is important to address the expected scale-dependence or independence. In the case that the small scale model used in the development stage is considered to not represent the commercial scale, confirmation of suitable control in pilot scale and/or initial commercial batches is generally appropriate. The need for data from pilot/commercial batches is influenced by the magnitude of the purge factor calculated from laboratory or pilot scale data, point of entry of the impurity, and knowledge of downstream process purge points.

If Options 3 and 4 cannot be justified, then a test for the impurity on the specification for a raw material, starting material or intermediate, or as an in-process control (Option 2) or drug substance (Option 1) at the acceptable limit should be included. For impurities introduced in the last synthetic step, an Option 1 control approach would be expected unless otherwise justified.

The application of "As Low As Reasonably Practicable" (ALARP) is not necessary if the level of the mutagenic impurity is below acceptable limits. Similarly, it is not necessary to demonstrate that alternate routes of synthesis have been explored.

In cases where control efforts cannot reduce the level of the mutagenic impurity to below the acceptable limit and levels are ALARP, a higher limit may be justified based on a risk/benefit analysis.

8.3 Considerations for Periodic Testing

The above options include situations where a test is recommended to be included in the specification, but where routine measurement for release of every batch may not be necessary. This approach, referred to as periodic or skip testing in ICH Q6A could also be called "Periodic Verification Testing." This approach may be appropriate when it can be demonstrated that processing subsequent to impurity formation/introduction clears the impurity. It should be noted that allowance of Periodic Verification Testing is contingent upon use of a process that is under a state of control (i.e., produces a quality product that consistently meets specifications and conforms to an appropriately established facility, equipment, processing, and operational control regimen). If upon testing, the level of the mutagenic impurity fails to meet the acceptance criteria established for the periodic test, the drug producer should immediately commence full testing (i.e., testing of every batch for the attribute specified) until the cause of the failure has been conclusively determined, corrective

action has been implemented, and the process is again documented to be in a state of control. As noted in ICH Q6A, regulatory authorities should be notified of a periodic verification test failure to evaluate the risk/benefit of previously released batches that were not tested.

8.4 Control of Degradation Products

For a potential degradation product that has been characterized as mutagenic, it is important to understand if the degradation pathway is relevant to the drug substance and drug product manufacturing processes and/or their proposed packaging and storage conditions. A well-designed accelerated stability study (e.g., 40°C/75% relative humidity, 6 months) in the proposed packaging, with appropriate analytical procedures is recommended to determine the relevance of the potential degradation product. Alternatively, well designed kinetically equivalent shorter term stability studies at higher temperatures in the proposed commercial package may be used to determine the relevance of the degradation pathway prior to initiating longer term stability studies. This type of study would be especially useful to understand the relevance of those potential degradation products that are based on knowledge of potential degradation pathways but not yet observed in the product.

Based on the result of these accelerated studies, if it is anticipated that the degradation product will form at levels approaching the acceptable limit under the proposed packaging and storage conditions, then efforts to control formation of the degradation product is expected. In these cases, monitoring for the drug substance or drug product degradation product in long term primary stability studies at the proposed storage conditions (in the proposed commercial pack) is expected unless otherwise justified. Whether or not a specification limit for the mutagenic degradation product is appropriate will generally depend on the results from these stability studies.

If it is anticipated that formulation development and packaging design options are unable to control mutagenic degradation product levels to less than the acceptable limit and levels are as low as reasonably practicable, a higher limit can be justified based on a risk/benefit analysis.

8.5 Lifecycle Management

This section is intended to apply to those products approved after the issuance of this guideline.

The quality system elements and management responsibilities described in ICH Q10 are intended to encourage the use of science-based and risk-based approaches at each lifecycle stage, thereby promoting continual improvement across the entire product lifecycle. Product and process knowledge should be managed from development through the commercial life of the product up to and including product discontinuation.

The development and improvement of a drug substance or drug product manufacturing process usually continues over its lifecycle. Manufacturing process performance, including the effectiveness of the control strategy, should be periodically evaluated. Knowledge gained from commercial manufacturing can be used to further improve process understanding and process performance and to adjust the control strategy.

Any proposed change to the manufacturing process should be evaluated for the impact on the quality of drug substance and drug product. This evaluation should be based on understanding of the manufacturing process and should determine if appropriate testing to analyze the impact of the proposed changes is required. Additionally, improvements in analytical procedures may lead to structural identification of an impurity. In those cases the new structure would be assessed for mutagenicity as described in this guideline.

Throughout the lifecycle of the product, it will be important to reassess if testing is recommended when intended or unintended changes occur in the process. This applies when there is no routine monitoring at the acceptable limit (Option 3 or Option 4 control approaches), or when applying periodic rather than batch-by-batch testing. This testing should be performed at an appropriate point in the manufacturing process.

In some cases, the use of statistical process control and trending of process measurements can be useful for continued suitability and capability of processes to provide adequate control on the impurity. Statistical process control can be based on process parameters that influence impurity formation or clearance, even when that impurity is not routinely monitored (e.g., Option 4).

All changes should be subject to internal change management processes as part of the quality system (ICH Q10). Changes to information filed and approved in a dossier should be reported to regulatory authorities in accordance with regional regulations and guidelines.

8.6 Considerations for Clinical Development

It is recognized that product and process knowledge increases over the course of development and therefore it is expected that data to support control strategies in the clinical development trial phases will be less than at the marketing registration phase. A risk-based approach based on process chemistry fundamentals is encouraged to prioritize analytical efforts on those impurities with the highest likelihood of being present in the drug substance or drug product. Analytical data may not be expected to support early clinical development when the likelihood of an impurity being present is low, but in a similar situation analytical data may be appropriate to support the control approach for the marketing application. It is also recognized that commercial formulation design occurs later in clinical development and therefore efforts associated with drug product degradation products will be limited in the earlier phases.

9. DOCUMENTATION

Information relevant to the application of this guideline should be provided at the following stages:

9.1 Clinical Trial Applications

- It is expected that the number of structures assessed for mutagenicity, and the collection of analytical data will both increase throughout the clinical development period.
- For Phase 1 studies of 14 days or less a description of efforts to mitigate risks of mutagenic impurities focused on Class 1, and Class 2 impurities and those in the cohort of concern as outlined in Section 7 should be included. For Phase 1 clinical trials greater than 14 days and for Phase 2a clinical trials additionally Class 3 impurities that require analytical controls should be included.
- For Phase 2b and Phase 3 clinical development trials, a list of the impurities assessed by (Q)SAR should be included, and any Class 1, 2 or 3 actual and potential impurities should be described along with plans for control. The *in silico* (Q)SAR systems used to perform the assessments should be described. The results of bacterial mutagenicity tests of actual impurities should be reported.
- Chemistry arguments may be appropriate instead of analytical data for potential impurities that present a low likelihood of being present as described in Section 8.6.

9.2 Common Technical Document (Marketing Application)

- For actual and potential process related impurities and degradation products where assessments according to this guideline are conducted, the mutagenic impurity classification and rationale for this classification should be provided:
  - This would include the results and description of *in silico* (Q)SAR systems used, and as appropriate, supporting information to arrive at the overall conclusion for Class 4 and 5 impurities.
  - When bacterial mutagenicity assays were performed on impurities, study reports should be provided for bacterial mutagenicity assays on impurities.
- Justification for the proposed specification and the approach to control should be provided (e.g., ICH Q11 example 5b, Ref. 12). For example, this information could include the acceptable intake, the location and sensitivity of relevant routine monitoring. For Option 3 and Option 4 control approaches, a summary of knowledge of the purge factor, and identification of factors providing control (e.g., process steps, solubility in wash solutions, etc.) is important.

NOTES

Note 1 The ICH M7 Guideline recommendations provide a state-of-the-art approach for assessing the potential of impurities to induce point mutations and ensure that such impurities are controlled to safe levels so that below or above the ICH Q3A/B qualification threshold no further qualification for mutagenic potential is required. This includes the initial use of (Q)SAR tools to predict bacterial mutagenicity. In cases where the amount of the impurity exceeds 1 mg daily dose for chronic administration, evaluation of genotoxic potential as recommended in ICH Q3A/B could be considered. In cases where the amount of the impurity is less than 1 mg, no further genotoxicity testing is required regardless of other qualification thresholds.

Note 2 To assess the mutagenic potential of impurities, a single bacterial mutagenicity assay can be carried out with a fully adequate protocol according to ICH S2(R1) and OECD 471 guidelines (Ref. 13 and 14). The assays are expected to be performed in compliance with Good Laboratory Practices (GLP) regulations; however, lack of full GLP compliance does not necessarily mean that the data cannot be used to support clinical trials and marketing authorizations. Such deviations should be described in the study report. For example, the test article may not be prepared or analyzed in compliance with GLP regulations. In some cases, the selection of bacterial tester strains may be limited to those proven to be sensitive to the identified alert. For impurities that are not feasible to isolate or synthesize or when compound quantity is limited, it may not be possible to achieve the highest test concentrations recommended for an ICH-compliant bacterial mutagenicity assay according to the current testing guidelines. In this case, bacterial mutagenicity testing could be carried out using a miniaturized assay format with proven high concordance to the ICH-compliant assay to enable testing at higher concentrations with justification.

Note 3 Tests to Investigate the *in vivo* Relevance of *in vitro* Mutagens (Positive Bacterial Mutagenicity)

In vivo test	Factors to justify choice of test as fit-for-purpose
Transgenic mutation assays	• For any bacterial mutagenicity positive. Justify selection of assay tissue/organ
Pig-a assay (blood)	• For directly acting mutagens (bacterial mutagenicity positive without S9)*
Micronucleus test (blood or bone marrow)	• For directly acting mutagens (bacterial mutagenicity positive without S9) and compounds known to be clastogenic*

Rat liver Unscheduled DNA Synthesis (UDS) test	• In particular for bacterial mutagenicity positive with S9 only
	• Responsible liver metabolite known
	  ○ to be generated in test species used
	  ○ to induce bulky adducts

Comet assay	• Justification needed (chemical class specific mode of action to form alkaline labile sites or single-strand breaks as preceding DNA damage that can potentially lead to mutations • Justify selection of assay tissue/organ
Others	• With convincing justification

*For indirect acting mutagens (requiring metabolic activation), adequate exposure to metabolite(s) should be demonstrated.

Note 4 Example of linear extrapolation from the TD_{50}

It is possible to calculate a compound-specific acceptable intake based on rodent carcinogenicity potency data such as TD_{50} values (doses giving a 50% tumor incidence equivalent to a cancer risk probability level of 1:2). Linear extrapolation to a probability of 1 in 100,000 (i.e., the accepted lifetime risk level used) is achieved by simply dividing the TD_{50} by 50,000. This procedure is similar to that employed for derivation of the TTC.

Calculation example: Ethylene oxide

TD_{50} values for ethylene oxide according to the Carcinogenic Potency Database are 21.3 mg/kg body weight/day (rat) and 63.7 mg/kg body weight/day (mouse). For the calculation of an acceptable intake, the lower (i.e., more conservative) value of the rat is used.

To derive a dose to cause tumors in 1 in 100,000 animals, divide by 50,000:

21.3 mg/kg ÷ 50,000 = 0.42 µg/kg

To derive a total human daily dose:

0.42 µg/kg/day x 50 kg body weight = 21.3 µg/person/day

Hence, a daily life-long intake of 21.3 µg ethylene oxide would correspond to a theoretical cancer risk of 10^{-5} and therefore be an acceptable intake when present as an impurity in a drug substance.

Alternative methods and published regulatory limits for cancer risk assessment

As an alternative of using the most conservative TD_{50} value from rodent carcinogenicity studies irrespective of its relevance to humans, an in-depth toxicological expert assessment of the available carcinogenicity data can be done in order to initially identify the findings (species, organ, etc.) with highest relevance to human risk assessment as a basis for deriving a reference point for linear extrapolation. Also, in order to better take into account directly the shape of the dose-response curve, a benchmark dose such as a Benchmark Dose Lower Confidence Limit 10% (BMDL10, an estimate of the lowest dose which is 95% certain to cause no more than a 10% cancer incidence in rodents) may be used instead of TD_{50} values as a numerical index for carcinogenic potency. Linear extrapolation to a probability of 1 in 100,000 (i.e., the accepted lifetime risk level used) is then achieved by simply dividing the BMDL10 by 10,000.

Compound-specific acceptable intakes can also be derived from published recommended values from internationally recognized bodies such as World Health Organization (WHO, International Program on Chemical Safety [IPCS] Cancer Risk Assessment Programme) and others using the appropriate 10^{-5} lifetime risk level. In general, a regulatory limit that is applied should be based on the most current and scientifically supported data and/or methodology.

Note 5 A compound-specific calculation of acceptable intakes for mutagenic impurities may be applied for mutagenic impurities (without carcinogenicity data) which are structurally similar to a chemically-defined class of known carcinogen. For example, factors that are associated with the carcinogenic potency of monofunctional alkyl chlorides have been identified (Ref. 15) and can be used to modify the safe acceptable intake of monofunctional alkyl chlorides, a group of alkyl chlorides commonly used in drug synthesis. Compared to multifunctional alkyl chlorides the monofunctional compounds are much less potent carcinogens with TD_{50} values ranging from 36 to 1810 mg/kg/day (n=15; epichlorohydrin with two distinctly different functional groups is excluded). A TD_{50} value of 36 mg/kg/day can thus be used as a still very conservative class-specific potency reference point for calculation of acceptable intakes for monofunctional alkyl chlorides. This potency level is at least ten-fold lower than the TD_{50} of 1.25 mg/kg/day corresponding to the default lifetime TTC (1.5 µg/day) and therefore justifies lifetime and less-than-lifetime daily intakes for monofunctional alkyl chlorides ten times the default ones.

Note 6 Establishing less-than-lifetime acceptable intakes for mutagenic impurities in pharmaceuticals has precedent in the establishment of the staged TTC limits for clinical development (Ref. 16). The calculation of less-than-lifetime Acceptable Intakes (AI) is predicated on the principle of Haber's rule, a fundamental concept in toxicology where concentration (C) x time (T) = a constant (k). Therefore, the carcinogenic effect is based on both dose and duration of exposure.

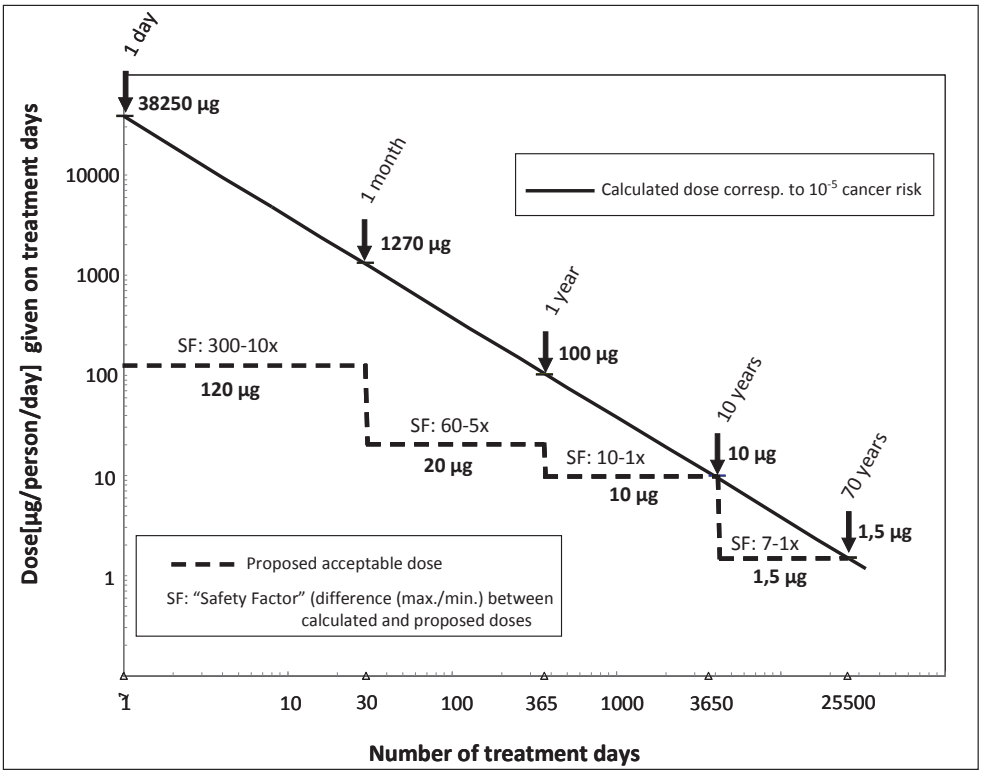

Figure 1: Illustration of calculated daily dose of a mutagenic impurity corresponding to a theoretical 1:100,000 cancer risk as a function of duration of treatment in comparison to the acceptable intake levels as recommended in Section 7.3.

The solid line in Figure 1 represents the linear relationship between the amount of daily intake of a mutagenic impurity corresponding to a 10^{-5} cancer risk and the number of treatment days. The calculation is based on the TTC level as applied in this guideline for life-long treatment i.e., 1.5 µg per person per day using the formula:

$$\text{Less-than-lifetime AI} = \frac{1.5\ \mu g \times (365\ \text{days} \times 70\ \text{years lifetime} = 25{,}550)}{\text{Total number of treatment days}}$$

The calculated daily intake levels would thus be 1.5 µg for treatment duration of 70 years, 10 µg for 10 years, 100 µg for 1 year, 1270 µg for 1 month and approximately 38.3 mg as a single dose, all resulting in the same cumulative intake and therefore theoretically in the same cancer risk (1 in 100,000).

The dashed step-shaped curve represents the actual daily intake levels adjusted to less-than-lifetime exposure as recommended in Section 7 of this guideline for products in clinical development and marketed products. These proposed levels are in general significantly lower than the calculated values thus providing safety factors that increase with shorter treatment durations.

The proposed accepted daily intakes are also in compliance with a 10^{-6} cancer risk level if treatment durations are not longer than 6 months and are therefore applicable in early clinical trials with volunteers/patients where benefit has not yet been established. In this case the safety factors as shown in the upper graph would be reduced by a factor of 10.

Note 7 **Table 4**: Examples of clinical use scenarios with different treatment durations for applying acceptable intakes

Scenario[1]	Acceptable Intake (µg/day)
Treatment duration of ≤ 1 month: e.g., drugs used in emergency procedures (antidotes, anesthesia, acute ischemic stroke), actinic keratosis, treatment of lice	120
Treatment duration of > 1-12 months: e.g., anti-infective therapy with maximum up to 12 months treatment (HCV), parenteral nutrients, prophylactic flu drugs (~ 5 months), peptic ulcer, Assisted Reproductive Technology (ART), pre-term labor, preeclampsia, pre-surgical (hysterectomy) treatment, fracture healing (these are acute use but with long half-lives)	20
Treatment duration of >1-10 years: e.g., stage of disease with short life expectancy (severe Alzheimer's), non-genotoxic anticancer treatment being used in a patient population with longer term survival (breast cancer, chronic myelogenous leukemia), drugs specifically labeled for less than 10 years of use, drugs administered intermittently to treat acute recurring symptoms[2] (chronic Herpes, gout attacks, substance dependence such as smoking cessation), macular degeneration, HIV[3]	10
Treatment duration of >10 years to lifetime: e.g., chronic use indications with high likelihood for lifetime use across broader age range (hypertension, dyslipidemia, asthma, Alzheimer's (except severe Alzheimer disease), hormone therapy (e.g., growth hormone, thyroid hormone, parathyroid hormone), lipodystrophy, schizophrenia, depression, psoriasis, atopic dermatitis, Chronic Obstructive Pulmonary Disease (COPD), cystic fibrosis, seasonal and perennial allergic rhinitis	1.5

[1] This table shows general examples; each example should be examined on a case-by-case basis. For example, 10 µg/day may be acceptable in cases where the life expectancy of the patient may be limited e.g., severe Alzheimer's disease, even though the drug use could exceed 10 year duration.

[2] Intermittent use over a period >10 years but based on calculated cumulative dose it falls under the >1-10 year category.

[3] HIV is considered a chronic indication but resistance develops to the drugs after 5-10 years and the therapy is changed to other HIV drugs.

GLOSSARY

Acceptable intake:

In the context of this guideline, an intake level that poses negligible cancer risk, or for serious/life-threatening indications where risk and benefit are appropriately balanced.

Acceptable limit:

Maximum acceptable concentration of an impurity in a drug substance or drug product derived from the acceptable intake and the daily dose of the drug.

Acceptance criterion:

Numerical limits, ranges, or other suitable measures for acceptance of the results of analytical procedures.

Control strategy:

A planned set of controls, derived from current product and process understanding that ensures process performance and product quality. The controls can include parameters and attributes related to drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control.

Cumulative intake:

The total intake of a substance that a person is exposed to over time.

Degradation Product: A molecule resulting from a chemical change in the drug molecule brought about over time and/or by the action of light, temperature, pH, water, or by reaction with an excipient and/or the immediate container/closure system.

DNA-reactive:

The potential to induce direct DNA damage through chemical reaction with DNA.

Expert knowledge:

In the context of this guideline, expert knowledge can be defined as a review of pre-existing data and the use of any other relevant information to evaluate the accuracy of an *in silico* model prediction for mutagenicity.

Genotoxicity:

A broad term that refers to any deleterious change in the genetic material regardless of the mechanism by which the change is induced.

Impurity:

Any component of the drug substance or drug product that is not the drug substance or an excipient.

Mutagenic impurity:

An impurity that has been demonstrated to be mutagenic in an appropriate mutagenicity test model, e.g., bacterial mutagenicity assay.

Periodic verification testing:

Also known as periodic or skip testing in ICH Q6A.

(Q)SAR and SAR:

In the context of this guideline, refers to the relationship between the molecular (sub) structure of a compound and its mutagenic activity using (Quantitative) Structure-Activity Relationships derived from experimental data.

Purge factor:

Purge reflects the ability of a process to reduce the level of an impurity, and the purge factor is defined as the level of an impurity at an upstream point in a process divided by the level of an impurity at a downstream point in a process. Purge factors may be measured or predicted.

Structural alert:

In the context of this guideline, a chemical grouping or molecular (sub) structure which is associated with mutagenicity.

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APPENDICES

Appendix 1: Scope Scenarios for Application of the ICH M7 Guideline

Scenario	Applies to Drug Substance	Applies to Drug Product	Comments
Registration of new drug substances and associated drug product	Yes	Yes	Primary intent of the M7 Guideline
Clinical trial applications for new drug substances and associated drug product	Yes	Yes	Primary intent of the M7 Guideline
Clinical trial applications for new drug substances for a anticancer drug per ICH S9	No	No	Out of scope of M7 Guideline
Clinical trial applications for new drug substances for an orphan drug	Yes	Yes	There may be exceptions on a case by case basis for higher impurity limits
Clinical trial application for a new drug product using an existing drug substance where there are no changes to the drug substance manufacturing process	No	Yes	Retrospective application of the M7 Guideline is not intended for marketed products unless there are changes made to the synthesis. Since no changes are made to the drug substance synthesis, the drug substance would not require reevaluation. Since the drug product is new, application of this guideline is expected.
A new formulation of an approved drug substance is filed	No	Yes	See Section 4.2
A product that is previously approved in a member region is filed for the first time in a different member region. The product is unchanged.	Yes	Yes	As there is no mutual recognition, an existing product in one member region filed for the first time in another member region would be considered a new product.
A new supplier or new site of the drug substance is registered. There are no changes to the manufacturing process used in this registered application.	No	No	As long as the synthesis of the drug substance is consistent with previously approved methods, then reevaluation of mutagenic impurity risk is not necessary. The applicant would need to demonstrate that no changes have been made to a previously approved process/product. See Section 4.1.

An existing product (approved after the issuance of ICH M7 with higher limits based on ICH S9) associated with an advanced cancer indication is now registered for use in a non-life threatening indication	Yes	Yes	Since the patient population and acceptable cancer risk have changed, the previously approved impurity control strategy and limits will require reevaluation. See Section 4.3.
New combination product is filed that contains one new drug substance and an existing drug substance	Yes (new drug substance) No (existing drug substance)	Yes	M7 would apply to the new drug substance. For the existing drug substance, retrospective application of M7 to existing products is not intended. For the drug product, this would classify as a new drug product so the guideline would apply to any new or higher levels of degradation products.

Appendix 2: Case Examples to Illustrate Potential Control Approaches

Case 1: Example of an Option 3 Control Strategy

An intermediate X is formed two steps away from the drug substance and impurity A is routinely detected in intermediate X. The impurity A is a stable compound and carries over to the drug substance. A spike study of the impurity A at different concentration levels in intermediate X was performed at laboratory scale. As a result of these studies, impurity A was consistently removed to less than 30% of the TTC-based limit in the drug substance even when impurity A was present at 1% in intermediate X. Since this intermediate X is formed only two steps away from the drug substance and the impurity A level in the intermediate X is relatively high, the purging ability of the process has additionally been confirmed by determination of impurity A in the drug substance in multiple pilot-scale batches and results were below 30% of the TTC-based limit. Therefore, control of the impurity A in the intermediate X with an acceptance limit of 1.0% is justified and no test is warranted for this impurity in the drug substance specification.

Case 2: Example of an Option 3 Control Strategy: Based on Predicted Purge from a Spiking Study Using Standard Analytical Methods

A starting material Y is introduced in step 3 of a 5-step synthesis and an impurity B is routinely detected in the starting material Y at less than 0.1% using standard analytical methods. In order to determine if the 0.1% specification in the starting material is acceptable, a purge study was conducted at laboratory scale where impurity B was spiked into starting material Y with different concentration levels up to 10% and a purge factor of > 500-fold was determined across the final three processing steps. This purge factor applied to a 0.1% specification in starting material Y would result in a predicted level of impurity B in the drug substance of less than 2 ppm. As this is below the TTC-based limit of 50 ppm for this impurity in the drug substance, the 0.1% specification of impurity B in starting material Y is

justified without the need for providing drug substance batch data on pilot scale or commercial scale batches.

Case 3: Example of an Option 2 and 4 Control Strategy: Control of Structurally Similar Mutagenic Impurities

The step 1 intermediate of a 5-step synthesis is a nitroaromatic compound that may contain low levels of impurity C, a positional isomer of the step 1 intermediate and also a nitroaromatic compound. The amount of impurity C in the step 1 intermediate has not been detected by ordinary analytical methods, but it may be present at lower levels. The step 1 intermediate is positive in the bacterial mutagenicity assay. The step 2 hydrogenation reaction results in a 99% conversion of the step 1 intermediate to the corresponding aromatic amine. This is confirmed via in-process testing. An assessment of purge of the remaining step 1 nitroaromatic intermediate was conducted and a high purge factor was predicted based on purge points in the subsequent step 3 and 4 processing steps. Purge across the step 5 processing step is not expected and a specification for the step 1 intermediate at the TTC-based limit was established at the step 4 intermediate (Option 2 control approach). The positional isomer impurity C would be expected to purge via the same purge points as the step 1 intermediate and therefore will always be much lower than the step 1 intermediate itself and therefore no testing is required and an Option 4 control strategy for impurity C can be supported without the need for any additional laboratory or pilot scale data.

Case 4: Example of an Option 4 Control Strategy: Highly Reactive Impurity

Thionyl chloride is a highly reactive compound that is mutagenic. This reagent is introduced in step 1 of a 5-step synthesis. At multiple points in the synthesis, significant amounts of water are used. Since thionyl chloride reacts instantaneously with water, there is no chance of any residual thionyl chloride to be present in the drug substance. An Option 4 control approach is suitable without the need for any laboratory or pilot scale data.

Implementation of Guideline:

Implementation of M7 is encouraged after publication; however, because of the complexity of the guideline, application of M7 is not expected prior to 18 months after ICH publication.

The following exceptions to the 18 month timeline apply.

1. Ames tests should be conducted according to M7 upon ICH publication. However, Ames tests conducted prior to publication of M7 need not be repeated.

2. When development programs have started phase 2b/3 clinical trials prior to publication of M7 these programs can be completed up to and including marketing application submission and approval, with the following exceptions to M7.
   - No need for two QSAR assessments as outlined in Section 6.
   - No need to comply with the scope of product impurity assessment as outlined in Section 5.
   - No need to comply with the documentation recommendations as outlined in Section 9.

3. Given the similar challenges for development of a commercial manufacturing process, application of the aspects of M7 listed above to new marketing applications that do not include Phase 2b/3 clinical trials would not be expected until 36 months after ICH publication of M7.

Appendix 3: Addendum to ICH M7

Application of the Principles of the ICH M7 Guideline to Calculation of Compound-Specific Acceptable Intakes

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AI	Acceptable Intakes
ATSDR	Agency for Toxic Substances & Disease Registry
BC	Benzyl Chloride
BCME	Bis(chloromethyl)ether
BUA	Biodegradable in water Under Aerobic conditions
CAC	Cancer Assessment Committee
CCRIS	Chemical Carcinogenesis Research Information System
CHL	Chinese Hamster Lung fibroblast cell line
CICAD	Concise International Chemical Assessment Document
CIIT	Chemical Industry Institute of Toxicology
CNS	Central Nervous System
CPDB	Carcinogenicity Potency Database
CYP	Cytochrome P-450
DMCC	Dimethylcarbamyl Chloride
DMS	Dimethyl Sulfate
DNA	Deoxyribose Nucleic Acid
EC	European Commission
ECHA	European Chemical Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPA	Environmental Protection Agency
EU	European Union
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
HSDB	Hazardous Substance Database
IARC	International Agency for Research on Cancer
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
JETOC	Japan Chemical Industry Ecology-Toxicology & Information Center
JRC	Joint Research Centre
LOAEL	Lowest-Observed Adverse Effect Level
MTD	Maximum Tolerated Dose
NA	Not applicable
NC	Not calculated; individual tumour type incidences not provided in WHO, 2002

NCI	National Cancer Institute
NOAEL	No-Observed Adverse Effect Level
NOEL	No-Observed Effect Level
NSRL	No Significant Risk Level
NTP	National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
PCE	Polychromatic Erythrocytes
PDE	Permissible Daily Exposure
RfC	Reference Concentration
ROS	Reactive Oxygen Species
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
SCE	Sister Chromatid Exchanges
SIDS	Screening Information Dataset
TBA	Tumor Bearing Animal
TD50	Chronic dose-rate in mg/kg body weight/day which would cause tumors in half of the animals at the end of a standard lifespan for the species taking into account the frequency of that tumor type in control animals
TTC-based	Threshold of Toxicological Concern-based
UDS	Unscheduled DNA Synthesis
UNEP	United Nations Environmental Programme
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

Introduction

The ICH M7 Guideline discusses the derivation of Acceptable Intakes (AIs) for mutagenic impurities with positive carcinogenicity data, (Section 7.2.1) and states: *"Compound-specific risk assessments to derive acceptable intakes should be applied instead of the TTC-based (Threshold of Toxicological Concern-based) acceptable intakes where sufficient carcinogenicity data exist. For a known mutagenic carcinogen, a compound-specific acceptable intake can be calculated based on carcinogenic potency and linear extrapolation as a default approach. Alternatively, other established risk assessment practices such as those used by international regulatory bodies may be applied either to calculate acceptable intakes or to use already existing values published by regulatory authorities."*

In this Addendum to ICH M7, AIs or Permissible Daily Exposures (PDEs) have been derived for a set of chemicals that are considered to be mutagens and carcinogens and are common in pharmaceutical manufacturing, or are useful to illustrate the principles for deriving compound-specific intakes described in ICH M7[1]. The set of chemicals include compounds in which the primary method used to derive AIs for carcinogens with a likely mutagenic mode of action is the "default approach" from ICH M7 of linear extrapolation from the calculated cancer potency estimate, the TD_{50}. Some chemicals that are mutagens and carcinogens (classified as Class 1 in ICH M7) may induce tumors through a non-mutagenic mode of action. Therefore, additional compounds are included to highlight alternative principles to deriving compound-specific intakes (i.e. PDE, see below). Other compounds (e.g., aniline) are included even though the available data indicates that they are non-mutagenic; nevertheless, the historical perception has been that they are genotoxic carcinogens.

ICH M7 states in Section 7.2.2: *"The existence of mechanisms leading to a dose response that is non-linear or has a practical threshold is increasingly recognized, not only for compounds that interact with non-DNA (Deoxyribose Nucleic Acid) targets but also for DNA-reactive compounds, whose effects may be modulated by, for example, rapid detoxification before coming into contact with DNA, or by effective repair of induced damage. The regulatory approach to such compounds can be based on the identification of a No-Observed Effect Level (NOEL) and use of uncertainty factors (see ICH Q3C(R5)…) to calculate a Permissible Daily Exposure (PDE) when data are available."*

[1] Some chemicals are included whose properties (including chemical reactivity, solubility, volatility, ionizability) allow efficient removal during the steps of most synthetic pathways, so that a specification based on an acceptable intake will not typically be needed.

Examples are included in this Addendum to illustrate assessments of mode of action for some Class 1 chemicals that justify derivation of a PDE calculated using uncertainty factors as described in ICH Q3C(R5) (Ref. 1). These chemicals include hydrogen peroxide, which induces oxidative stress, and aniline which induces tumors secondary to hemosiderosis as a consequence of methemoglobinemia.

It is emphasized that the AI or PDE values presented in this Addendum address carcinogenic risk. Other considerations, such as quality standards, may affect final product specifications. For example, the ICH M7 guidance (Section 7.2.2) notes that when calculating acceptable intakes from compound-specific risk assessments, an upper limit would be 0.5%, or, for example, 500 µg in a drug with a maximum daily dose of 100 mg.

Methods

The general approach used in this addendum for deriving AIs included a literature review, selection of cancer potency estimate [TD_{50}, taken from the CPDB (Carcinogenicity Potency Database (Ref. 2), or calculated from published studies using the same method as in the CPDB] and ultimately calculation of an appropriate AI or PDE in cases with sufficient evidence for a threshold mode of action (see Section 3). The literature review focused on data relating to exposure of the general population (i.e., food, water, and air), mutagenicity/genotoxicity, and carcinogenicity. Based on the description of DNA-reactive mutagens in ICH M7, results from the standard bacterial reverse mutation assay (Ames test) were used as the main criterion for determining that a chemical was mutagenic. Other genotoxicity data, especially in vivo, were considered in assessing a likely mode of action for tumor induction. Any national or international regulatory values for acceptable exposure levels (e.g., US EPA, US FDA, EMA, ECHA, WHO) are described in the compound-specific assessments. Toxicity information from acute, repeat-dose, reproductive, neurological, and developmental studies was not reviewed in depth except to evaluate observed changes that act as a carcinogenic precursor event (e.g., irritation/inflammation, or methemoglobinemia).

1. Standard Method

1.1 Linear Mode of Action and Calculation of AI

Note 4 of ICH M7 states: *"It is possible to calculate a compound-specific acceptable intake based on rodent carcinogenicity potency data such as TD_{50} values (doses giving a 50% tumor incidence equivalent to a cancer risk probability level of 1:2). Linear extrapolation to a probability of 1 in 100,000 (i.e., the accepted lifetime risk level used) is achieved by simply dividing the TD_{50} by 50,000. This procedure is similar to that employed for derivation of the TTC."*

Thus, linear extrapolation from a TD_{50} value was considered appropriate to derive an AI for those Class 1 impurities (known mutagenic carcinogens) with no established "threshold mechanism", that is, understanding of a mode of action that results in a non-linear dose-response curve. In many cases, the carcinogenicity data were available from the CPDB; the conclusions were based either on the opinion of the original authors of the report on the carcinogenicity study ("author opinion" in CPDB) or on the conclusions of statistical analyses provided in the CPDB. When a pre-calculated TD_{50} value was identified in the CPDB for a selected chemical, this value was used to calculate the AI; the relevant carcinogenicity data were not reanalyzed and the TD_{50} value was not recalculated.

If robust data were available in the literature but not in the CPDB, then a TD_{50} was calculated based on methods described in the CPDB (Ref. 3). The assumptions for animal body weight, respiratory volume, and water consumption for calculation of doses were adopted from ICH Q3C and ICH Q3D (Ref. 1, 4).

1.2 Selection of Studies

The quality of studies in the CPDB is variable, although the CPDB does impose criteria for inclusion such as the proportion of the lifetime during which test animals were exposed. For the purposes of this Addendum additional criteria were applied when studies were of lesser quality. Studies of lesser quality are defined here as those where one or more of the following scenarios were encountered:
< 50 animals per dose per sex;
< 3 dose levels;
Lack of concurrent controls;
Intermittent dosing (< 5 days per week);
Dosing for less than lifetime.

The more robust studies were generally used to derive limits. However studies that did not fulfill all of the above criteria were in some cases considered adequate for derivation of an AI when other aspects of the study were robust, for example when treatment was for 3 days per week (e.g., benzyl chloride) but there was evidence that higher doses would not have been tolerated, i.e., a Maximum Tolerated Dose (MTD) as defined by the National Toxicology Program (NTP) or ICH S1C(R2) (Ref. 5) was attained. Calculations of potency take intermittent or less-than-lifetime dosing such as that for benzyl chloride into account; for example, in the CPDB the dose levels shown have been adjusted to reflect the estimated daily dose levels, such that the daily dose given 3 times per week is multiplied by 3/7 to give an average daily dose; a comparable adjustment is made if animals are treated for less than 24 months. Use of less robust data can sometimes be considered acceptable when no more complete data exist, given the highly conservative nature of the risk assessment in which TD_{50} was linearly extrapolated to a 1 in 100,000 excess cancer risk. In these cases, the rationale supporting the basis for the recommended approach is provided in the compound-specific assessments.

1.3 Selection of Tumor and Site

The lowest TD_{50} of a particular organ site for an animal species and sex was selected from the most robust studies. When more than one study exists, the CPDB provides a calculated harmonic mean TD_{50}, but in this Addendum the lowest TD_{50} was considered a more conservative estimate. Data compiled as "all Tumor Bearing Animals" (TBA) were not considered in selecting an appropriate TD_{50} from the CPDB; mixed tumor types (e.g., adenomas and carcinomas) in one tissue (e.g., liver) were used where appropriate as this often gives a more sensitive potency estimate.

1.4 Route of Administration

Section 7.5 of ICH M7 states: "The *above risk approaches described in Section 7 are applicable to all routes of administration and no corrections to acceptable intakes are generally warranted. Exceptions to consider may include situations where data justify route-specific concerns that should be evaluated case-by-case."*

In this Addendum, when robust data were available from carcinogenicity studies for more than one route, and the tumor sites did not appear to be route-specific, the TD_{50} from the route with the lowest TD_{50} value was selected for the AI calculation and is thus usually considered suitable for all routes. Exceptions may be necessary case by case; for example, in the case of a potent site-of-contact carcinogen a route-specific AI or PDE might be necessary. Other toxicities such as irritation might also limit the AI for a certain route, but only tumorigenicity is considered in this Addendum similar to M7. Here, if tumors were considered site-specific (e.g., inhalation exposure resulting in respiratory tract tumors with no tumors at distal sites) and the TD_{50} was lower than for other routes, then a separate AI was developed for that route (e.g., dimethyl carbamoyl chloride, hydrazine).

1.5 Calculation of AI from the TD_{50}

Calculating the AI from the TD_{50} is as follows (see Note 4 of ICH M7 for example):

$AI = TD_{50} / 50{,}000 \times 50\ kg$

The weight adjustment assumes an arbitrary adult human body weight for either sex of 50 kg. This relatively low weight provides an additional safety factor against the standard weights of 60 kg or 70 kg that are often used in this type of calculation. It is recognized that some adult patients weigh less than 50 kg; these patients are considered to be accommodated by the inherent conservatism (i.e., linear extrapolation of the most sensitive organ site) used to determine an AI.

2. Consideration of Alternative Methods for Calculation of AI

2.1 Human relevance of tumors

Note 4 of ICH M7 states: *"As an alternative of using the most conservative TD_{50} value from rodent carcinogenicity studies irrespective of its relevance to humans, an in-depth toxicological expert assessment of the available carcinogenicity data can be done in order to initially identify the findings (species, organ, etc.) with highest relevance to human risk assessment as a basis for deriving a reference point for linear extrapolation."*

Human relevance of the available carcinogenicity data was considered for deriving AIs. Effects in rodents associated with toxicities that occur with a non-linear dose response are not relevant to humans at the low, non-toxic concentrations associated with a pharmaceutical impurity. For example, in the case of *p*-chloroaniline, the most sensitive site for tumor induction was the spleen, but these tumors were associated with hemosiderosis, considered to be a mode of action with a non-linear dose response, and thus not relevant to humans at low doses that do not induce hemosiderosis. In the case of *p*-chloroaniline, liver tumors, with a higher TD_{50}, were used for the linear extrapolation to calculate the AI because a mutagenic mode of action could not be ruled out for liver tumors. A second category of tumors considered not to be relevant to humans is tumors associated with a rodent-specific mode of action e.g., methyl chloride, with species difference in metabolism.

2.2 Published regulatory limits

Note 4 of ICH M7 also states: *"Compound-specific acceptable intakes can also be derived from published recommended values from internationally recognized bodies such as World*

Health Organization (WHO, International Programme on Chemical Safety (IPCS) Cancer Risk Assessment Programme) and others using the appropriate 10^{-5} lifetime risk level. In general, a regulatory limit that is applied should be based on the most current and scientifically supported data and/or methodology."

In this Addendum, available regulatory limits are described (omitting occupational health limits as they are typically regional and may use different risk levels). However the conservative linear extrapolation from the TD_{50} was generally used as the primary method to derive the AI, as the default approach of ICH M7, and for consistency across compounds. It is recognized that minor differences in methodology for cancer risk assessment can result in different recommended limits (for example adjusting for body surface area in calculations), but the differences are generally quite small when linear extrapolation is the basis of the calculation.

3. Non-linear (Threshold) Mode of Action and Calculation of PDE

ICH M7 states in Section 7.2.2: *"The existence of mechanisms leading to a dose response that is non-linear or has a practical threshold is increasingly recognized, not only for compounds that interact with non-DNA targets but also for DNA-reactive compounds, whose effects may be modulated by, for example, rapid detoxification before coming into contact with DNA, or by effective repair of induced damage. The regulatory approach to such compounds can be based on the identification of a No-Observed Effect Level (NOEL) and use of uncertainty factors (see ICH Q3C(R5)) to calculate a Permissible Daily Exposure (PDE) when data are available."*

An example of a DNA-reactive chemical for which a threshold has been proposed for mutagenicity *in vitro* and *in vivo* is ethyl methane sulfonate (Ref. 6, 7). A PDE calculation using uncertainty factors, instead of linear extrapolation is appropriate in such cases where a threshold has been established.

This threshold approach was considered appropriate in the compound-specific assessments for carcinogens with modes of action (Section 2.1) that lack human relevance at low doses, based upon their association with a non-linear dose response for tumor induction:

Chemicals that induce methemoglobinemia, hemosiderin deposits in tissues such as spleen, and subsequent inflammation and tumors (e.g., aniline and related compounds);

Supporting information includes evidence that mutagenicity was not central to the mode of action, such as weak evidence for mutagenicity e.g., aniline; and/or lack of correlation between sites or species in which *in vivo* genotoxicity (such as DNA adducts) and tumor induction were seen.

Chemicals that induce tumors associated with local irritation/inflammation (such as rodent forestomach tumors) and are site-of-contact carcinogens may be considered not relevant to human exposure at low, non-irritating concentrations as potential impurities in pharmaceuticals (e.g., benzyl chloride);

Chemicals that act through oxidative damage, so that deleterious effects do not occur at lower doses since abundant endogenous protective mechanisms exist, (e.g., hydrogen peroxide).

Acceptable exposure levels for carcinogens with a threshold mode of action were established by calculation of PDEs. The PDE methodology is further explained in ICH Q3C(R5) (Ref. 1) and ICH Q3D (Ref. 4).

4. Acceptable Limit Based on Exposure in the Environment, e.g., in the Diet

As noted in ICH M7 Section 7.5, *"Higher acceptable intakes may be justified when human exposure to the impurity will be much greater from other sources e.g., food, or endogenous metabolism (e.g., formaldehyde)."*

For example, formaldehyde is not a carcinogen orally, so that regulatory limits have been based on non-cancer endpoints. Health Canada (Ref. 8), WHO IPCS (Ref. 9) and US Environmental Protection Agency (EPA) (Ref. 10) recommend an oral limit of 0.2 mg/kg/day, or 10 mg/day for a 50 kg person.

References

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9. World Health Organization (WHO). International Programme on Chemical Safety (IPCS). 2002. Concise International Chemical Assessment Document 40. Formaldehyde. [Online]. Available from: URL: http://www.who.int/ipcs/publications/cicad/en/cicad40.pdf

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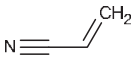
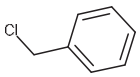
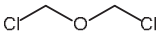
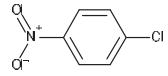
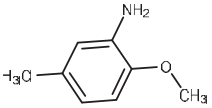
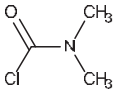
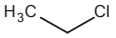
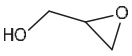
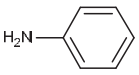
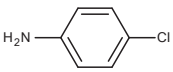
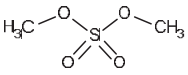
Acceptable Intakes (AIs) or Permissible Daily Exposures (PDEs)

Compound	CAS#	Chemical Structure	AI or PDE (µg/day)	Comment
Linear extrapolation from TD_{50}				
Acrylonitrile	107-13-1		6	TD_{50} linear extrapolation
Benzyl Chloride	100-44-7		41	TD_{50} linear extrapolation
Bis(chloromethyl)ether	542-88-1		0.004	TD_{50} linear extrapolation
1-Chloro-4-nitrobenzene	100-00-5		117	TD_{50} linear extrapolation
p-Cresidine	120-71-8		45	TD_{50} linear extrapolation
Dimethylcarbamoyl chloride	79-44-7		5 0.6 (Inhalation)*	TD_{50} linear extrapolation
Ethyl chloride	75-00-3		1,810	TD_{50} linear extrapolation
Glycidol	556-52-5		4	TD_{50} linear extrapolation
Hydrazine	302-01-2	$H_2N—NH_2$	39 0.2 (Inhalation)*	TD_{50} linear extrapolation
Methyl Chloride	74-87-3	$Cl\text{-}CH_3$	1,361	TD_{50} linear extrapolation
Threshold-based PDE				
Aniline Aniline HCl	62-53-3 142-04-1		720	PDE based on threshold mode of action (Hemosiderosis)
Endogenous and/or Environmental Exposure				
Hydrogen peroxide	7722-84-1	HO—OH	68,000 or 0.5% whichever is lower	68 mg/day is 1% of estimated endogenous production
Other Cases				

Compound	CAS#	Chemical Structure	AI or PDE (µg/day)	Comment
p-Chloroaniline *p*-Chloroaniline HCl	106-47-8 20265-96-7		34	AI based on liver tumors for which mutagenic mode of action cannot be ruled out
Dimethyl Sulfate	77-78-1		1.5	Carcinogenicity data available, but inadequate to derive AI. Default to TTC

*Route specific limit

Acrylonitrile (CAS# 107-13-1)

Potential for human exposure

No data are available for exposure of the general population.

Mutagenicity/Genotoxicity

Acrylonitrile is mutagenic and genotoxic *in vitro* and potentially positive *in vivo*.

The World Health Organization (WHO) Concise International Chemical Assessment Document (CICAD, Ref. 1), provided a thorough risk assessment of acrylonitrile. In this publication, oxidative metabolism was indicated as a critical step for acrylonitrile to exert genotoxic effects, implicating cyanoethylene oxide as a DNA-reactive metabolite. A detailed review of genotoxicity testing in a range of systems is provided (Ref. 1) with references, so only a few key conclusions are summarized here.

Acrylonitrile is mutagenic in:
Microbial reverse mutation assay (Ames) in *Salmonella typhimurium* TA 1535 and TA 100 only in the presence of rat or hamster S9 and in several *Escherichia coli* strains in the absence of metabolic activation;
Human lymphoblasts and mouse lymphoma cells, reproducibly with S9, in some cases without S9;
Splenic T cells of rats exposed *via* drinking water.

In vivo genotoxicity studies are negative or inconclusive, and reports of DNA binding are consistently positive in liver, but give conflicting results in brain.

Carcinogenicity

Acrylonitrile is classified by IARC as a Group 2B carcinogen, possibly carcinogenic to humans (Ref. 2).

Acrylonitrile is a multi-organ carcinogen in mice and rats, with the brain being the primary target organ in rat. There are four oral carcinogenicity studies cited in the CPDB (Ref. 3) and the results from three additional oral studies are summarized in Ref. 1. Of these seven studies only one is negative but this study tested only a single dose administered for short duration (Ref. 4).

The NCI/NTP (National Cancer Institute) study in the CPDB of acrylonitrile in mice (Ref. 5) was selected for derivation of the oral AI, based on robust study design and the most conservative TD_{50} value. In this 2 year-study, 3 doses of acrylonitrile were administered by oral gavage to male and female mice. There were statistically significant increases in tumors of the Harderian gland and forestomach.

In the 1980 study of Quast *et al* (Ref. 6), cited in the CPDB as a report from Dow Chemical, it appears that the most sensitive TD_{50} is for astrocytomas in female rats (5.31 mg/kg/day). However, this same study was later described in detail (Ref. 7) and the calculated doses in that published report are higher than those listed in the CPDB. Quast (Ref. 7) describes the derivation of doses in mg/kg/day from the drinking water concentrations of 35, 100 and 300 ppm, adjusting for body weight and the decreased water consumption in the study. The TD_{50} for astrocytomas derived from these numbers is 20.2 mg/kg/day for males and 20.8 for

females, in contrast to the calculated values in the CPDB of 6.36 and 5.31 mg/kg/day. (The TD_{50}'s calculated from the dose estimates by Quast (Ref. 7) for forestomach tumors are also higher than those in the CPDB based on the same study, as shown in the Table below). Central Nervous System (CNS), tumors are described (Ref. 7), but the most sensitive TD_{50} was for stomach tumors, as shown in the Table below.

Studies considered less robust included three rat drinking water studies. The largest study (Ref. 8) included five acrylonitrile treated groups with 100 animals per dose and 200 control animals, but serial sacrifices of 20 animals per treatment group occurred at 6, 12, 18 and 24 months. Data summaries by WHO (Ref. 1) and by US EPA (Ref. 9) present tumor incidence based on data from all time points combined. Therefore, the incidence of tumors reported may be an underestimate of the total tumors that would be observed if all animals were kept on study for 2 years. Two studies (Ref. 10, 11) each had only two dose levels and individual tumor types are not reported (Ref. 1), although tumors of stomach, Zymbal gland and brain were observed.

Acrylonitrile has also been studied by the inhalation route. Fifty rats per sex per dose were exposed for 2 years to acrylonitrile, and brain tumors were observed (Ref. 12). This study however, tested only 2 dose levels. The other inhalation studies were deficient in number of animals per group, duration of exposure, or administration of a single dose, although brain tumors were observed.

Acrylonitrile – Details of carcinogenicity studies

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/type/sex	TD_{50} (mg/kg/d)
Ref. 5*	50 B6C3F1 Mice (F)	2 years Gavage	50	**3:** 1.79;7.14; 14.3 mg/kg/d	Forestomach	6.77^{+}
	50 B6C3F1 Mice (M)	2 years Gavage	50	**3:** 1.79;7.14; 14.3 mg/kg/d	Forestomach	5.92^{+}
Ref. 6	~50 SD Spartan rats (F)	2 years Drinking water	~80	**3:** 2.00;5.69; 15.4 mg/kg/d	Astrocytoma	5.31^{++} (20.8)
	~50 SD Spartan rats (M)	2 years Drinking water	~80	**3:** 1.75;4.98; 14.9 mg/kg/d	Stomach, non-glandular	6.36^{++} (9.0)
Ref 7 (report of Ref. 6)	~50 female SD Spartan rats	2 years Drinking water	~80	**3:** 4.4;10.8; 25 mg/kg/d	Stomach, non-glandular	19.4
	~50 SD male Spartan rats	2 years Drinking water	~80	**3:** 3.4;8.5; 21.3 mg/kg/d	Stomach, non-glandular	9.0
Ref. 8¥	100 male rats	~2 years Drinking water	~200	**5:** 0.1-8.4 mg/kg/d	Brain astrocytoma	$(22.9)^{+}$

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/type/sex	TD_{50} (mg/kg/d)
	100 female rats	~2 years Drinking water	~200	**5:** 0.1-10.9 mg/kg/d	Brain astrocytoma	$(23.5)^{+}$
Ref. 11[¥]	100/sex Rats	19-22 mo Drinking water	~98	**2:** ~0.09; 7.98 mg/kg/d	Stomach, Zymbal's gland, brain, spinal cord	NC
Ref. 10[¥]	50/sex Rats	18 mo Drinking water	No	**2:** 14;70 mg/kg/d	Brain, Zymbal's gland, forestomach	NC[^]
Ref. 13	20 male CD rats	2 years Drinking water	No	**3:** 1; 5; 25 mg/kg/d	Zymbal's gland	30.1
Ref. 4	40/sex SD rats	1 year 3d/wk Gavage	75/sex	**1:** 1.07 mg/kg/d	Neg in both sexes	NA
Ref. 12	100/sex SD Spartan rat	2 years 6 h/d; 5d/wk Inhalation	~100	**2:** M: 2.27; 9.1 F: 3.24; 13.0 mg/kg/d	Brain Astrocytoma Male	32.4
Ref. 4	30/sex SD rats	1 year 5d/wk Inhalation	30	**4:** M: 0.19; 0.38; 0.76; 1.52 F: 0.27;0.54;1.0; 2.17 mg/kg/d	Brain glioma Male	19.1
Ref. 4	54 female SD rats	2 years 5d/wk Inhalation	60	**1:** 11.1 mg/kg/d	Brain glioma	$(132)^{\Psi}$

Studies listed are in CPDB (Ref. 3) unless otherwise noted.
The TD_{50} values represent the TD_{50} from the most sensitive tumor site.
TD_{50} values in parentheses are considered less reliable as explained in footnotes.
[*]Carcinogenicity study selected for AI calculation; in CPDB.
[^]NC= Not calculated as individual tumor type incidences not provided in WHO (Ref. 1).
[+]TD_{50} calculated based on astrocytoma incidence implied as most significant site by WHO (Ref. 1). Serial sampling reduced number of animals exposed for 2 years, so tumor incidences may be underestimates.
[++]Taken from the CPDB. Note that based on the dose calculations by the author (Ref. 7) the TD_{50} for astrocytomas and stomach tumors in Spartan rats (20.8 and 9.0) are higher than those in the CPDB.
NA= Not applicable.
[¥] Not in CPDB. Summarized in Refs. 1 and 9.
[Ψ] Single dose-level study.

Mode of action for carcinogenicity

Although the mechanism of carcinogenesis remains inconclusive, a contribution of DNA interaction cannot be ruled out (Ref. 1). CNS tumors were seen in multiple carcinogenicity

studies in rats, in addition to forestomach tumors; forestomach tumors were also the most sensitive tumor type in mice.

Forestomach tumors are associated with local irritation and inflammation, and Quast (Ref. 7) notes the typical association between these tumors in rats and hyperplasia and/or dyskeratosis, with other inflammatory and degenerative changes. Forestomach tumors in rodents administered high concentrations orally, a type of site-of-contact effect, may not be relevant to human exposure at low concentrations that are non-irritating (Ref. 14). Acrylonitrile is not only a site-of-contact carcinogen. Tumors were seen in the CNS, in addition to tissues likely to be exposed directly such as the gastrointestinal tract and tongue. Forestomach tumors were seen after administration of acrylonitrile to rats in drinking water, and to mice by gavage. The AI for acrylonitrile was derived based on mouse forestomach tumors.

Regulatory and/or published limits

The US EPA (Ref. 9) calculated an oral slope factor of 0.54 /mg/kg/day and a drinking water limit of 0.6 µg/L at the 1/100,000 risk level, based on the occurrence of multi-organ tumors in a drinking water study in rats. This drinking water limit equates to a daily dose of ~1 µg/day for a 50 kg human.

Acceptable intake (AI)

Rationale for selection of study for AI calculation

Both inhalation and oral studies (gavage and drinking water) are available. Tumors of the CNS were seen by both routes of administration, and acrylonitrile is rapidly absorbed *via* all routes of exposure and distributed throughout examined tissues (Ref. 1), so that a specific inhalation AI was not considered necessary. All of the carcinogenicity studies that were used by the US EPA (Ref. 9) in the derivation of the drinking water limit for acrylonitrile were reviewed when selecting the most robust carcinogenicity study for the derivation of an AI. The NCI/NTP study (Ref. 5) was selected to calculate the AI based on the TD_{50} derived from administering acrylonitrile by oral gavage to male and female mice since the tumor type with the lowest TD_{50} was forestomach tumors in male mice, with a TD_{50} value of 5.92 mg/kg/day. As discussed in the Methods Section 2.2, linear extrapolation from the TD_{50} was used here to derive the AI, and it is expected that minor differences in methodology can result in different calculated limits; thus the AI calculated below for potential pharmaceutical impurities is slightly higher than that derived by US EPA (Ref. 9) for drinking water.

Calculation of AI

Lifetime AI = TD_{50}/50,000 x 50kg

Lifetime AI = 5.92 (mg/kg/day)/50,000 x 50 kg

Lifetime AI = 5.9 µg/day (6 µg/day)

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Aniline (CAS# 62-53-3) and Aniline Hydrochloride (CAS# 142-04-1)

Potential for human exposure

Aniline occurs naturally in some foods (i.e., corn, grains, beans, and tea), but the larger source of exposure is in industrial settings.

Mutagenicity/genotoxicity

Aniline is not mutagenic in the microbial reverse mutation assay (Ames) in *Salmonella*. Aniline is included in this Addendum because of the historical perception that aniline is a genotoxic carcinogen, since some *in vitro* and *in vivo* genotoxicity tests are positive.

Aniline is not mutagenic in the 5 standard strains of *Salmonella* or in *E.Coli* WP2 *uvrA,* with or without S9 (Ref. 1, 2, 3, 4, 5, 6, 7, 8).

Aniline was positive in the mouse lymphoma L5178Y cell *tk* assay with and without S9 at quite high concentrations, such as 0.5 to 21 mM (Ref. 9, 10, 11).

Chromosomal aberration tests gave mixed results, with some negative reports and some positive results in hamster cell lines at very high, cytotoxic concentrations, e.g., about 5 to 30 mM, with or without S9 metabolic activation (Ref. 1, 12, 13, 14, 15).

In vivo, chromosomal aberrations were not increased in the bone marrow of male CBA mice after two daily intraperitoneal (i.p.) doses of 380 mg/kg (Ref. 16), but a small increase in chromosomal aberrations 18 h after an oral dose of 500 mg/kg to male PVR rats was reported (Ref. 17).

Most studies of micronucleus induction are positive in bone marrow after oral or i.p. treatment of mice (Ref. 18, 19, 20, 21) or rats (Ref. 17, 22), and most commonly at high doses, above 300 mg/kg. Dietary exposure to 500, 1000 and 2000 ppm for 90 days was associated with increases in micronuclei in peripheral blood of male and female B6C3F1 mice (Ref. 23).

In vivo, a weak increase in Sister Chromatid Exchanges (SCE), reaching a maximum of 2-fold increase over the background, was observed in the bone marrow of male Swiss mice 24 h after a single i.p. dose of 61 to 420 mg/kg aniline (Ref. 24, 25). DNA strand breaks were not detected in the mouse bone marrow by the alkaline elution assay in this study.

Carcinogenicity

Aniline is classified by IARC as Group 3, not classifiable as to its carcinogenicity in humans (Ref. 4).

Bladder cancers in humans working in the dye industry were initially thought to be related to aniline exposure but were later attributed to exposures to intermediates in the production of aniline dyes, such as β-naphthylamine, benzidine, and other amines.

The Chemical Industry Institute of Toxicology (CIIT, Ref. 26) performed a study in which aniline hydrochloride was administered in the diet for 2 years to CD-F rats (130 rats/sex/group) at levels of 0, 200, 600, and 2000 ppm. An increased incidence of primary splenic sarcomas was observed in male rats in the high dose group only. This study was selected for derivation of the PDE for aniline based on the robust study design with 3 dose groups and a large group size (130/sex/group).

The results of the CIIT study are consistent with those of the dietary study by the US National Cancer Institute (Ref. 27) of aniline hydrochloride in which male rats had increases in hemangiosarcomas in multiple organs including spleen, and a significant dose-related trend in incidence of malignant pheochromocytoma. In mice (Ref. 27), no statistically significant increase in any type of tumor was observed at very high doses.

Aniline itself did not induce tumors in rats when tested in a less robust study design (Ref. 28).

Aniline and Aniline HCl – Details of carcinogenicity studies

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/type/sex	TD_{50} (mg/kg/d)
Ref. 26* Aniline HCl	130/sex/ group, CD-F rats	2 years Diet	130	**3:** 200, 600 and 2000 ppm in diet (M; 7.2; 22; 72 mg/kg/d)	Spleen sarcoma (high dose). NOEL at low dose	Not reported
Ref. 27** Aniline HCl	50/sex/group, F344 rats	103 weeks (107-110 wk study) Diet	50	**2:** 3000 and 6000 ppm in diet (F: 144;268 M: 115;229 mg/kg/d)	Spleen hemangio-sarcoma/Male	160 (Male)
Ref. 27** Aniline HCl	50/sex/group B6C3F1 mice	103 weeks (107-110 wk study) Diet	50	**2:** 6000 and 12000 ppm in diet (F: 741;1500 M: 693;1390 mg/kg/d)	Negative	NA
Ref. 28** Aniline	10-18/group, male Wistar rats	80 weeks Diet	Yes	**3:** 0.03, 0.06 and 0.12% in diet (15;30;60 mg/kg/d)	Negative	NA

*Carcinogenicity study selected for PDE calculation. Not in CPDB.
**Taken from CPDB (Ref. 29). The TD_{50} values represent the TD_{50} from the most sensitive tumor site.
NA = Not applicable

Mode of action for carcinogenicity

In animal studies, aniline caused methemoglobinemia and hemolysis at high doses, the latter of which could indirectly lead to increases in micronuclei by inducing erythropoiesis (Ref. 19, 30, 31). Micronuclei are induced in both rats and mice, while aniline-induced tumors are seen in rats but not mice, adding to the evidence that genotoxicity is not key to the mode of action for aniline-induced tumors.

Aniline-induced toxicity in the spleen appears to be a contributory factor for its carcinogenicity *via* free radical formation and tissue injury (Ref. 32). High doses (>10 mg/kg) of aniline lead to iron accumulation in the spleen resulting from the preferential binding of aniline to red blood cells and damaged cells accumulating in the spleen. Iron-mediated oxidative stress in the spleen appears to induce lipid peroxidation, malondialdehyde-protein adducts, protein oxidation, and up-regulation of Transforming Growth Factor-β 1, all of which

have been detected in the rat spleen following aniline exposure (Ref. 33). Increased oxidative stress may be a continual event during chronic exposure to aniline and could contribute to the observed cellular hyperplasia, fibrosis, and tumorigenesis in rats (Ref. 32, 34). The lack of tumorigenicity in mice may be due to less severe toxicity observed in spleen compared to that in rats (Ref. 17, 35).

In support of this toxicity-driven mode of action for carcinogenicity, the dose response for aniline-induced tumorigenicity in rats is non-linear (Ref. 36). When considering the NCI and CIIT studies which both used the same rat strain, no tumors were observed when aniline hydrochloride was administered in the diet at a concentration of 0.02% (equal to approximately 7.2 mg/kg/day aniline in males). This, together with studies evaluating the pattern of accumulation of bound radiolabel derived from aniline in the spleen (Ref. 37) support the conclusion that a threshold exists for aniline carcinogenicity (Ref. 36). The weight of evidence supports the conclusion that these tumors do not result from a primary mutagenic mode of action (Ref. 38).

Regulatory and/or published limits

The US EPA (Ref. 39) outlines a quantitative cancer risk assessment for aniline based on the CIIT study (Ref. 26) and use of a linearised multistage. The resulting cancer potency slope curve was 0.0057/mg/kg/day and the dose associated with a 1 in 100,000 lifetime cancer risk is calculated to be 120 µg/day. However, the assessment states that this procedure may not be the most appropriate method for the derivation of the slope factor as aniline accumulation in the spleen is nonlinear (Ref. 39). Minimal accumulation of aniline and no hemosiderosis is observed at doses below 10 mg/kg and as already described, hemosiderosis may be important in the induction of the splenic tumors observed in rats.

Permissible daily exposure (PDE)

It is considered inappropriate to base an AI for aniline on linear extrapolation for spleen tumors observed in rats, since these have a non-linear dose response, aniline is not mutagenic, and genotoxicity is not central to the mode of action of aniline-induced carcinogenicity. The PDE is derived using the process defined in ICH Q3C (Ref. 40).

Rationale for selection of study for PDE calculation

Data from the CIIT 2-year rat carcinogenicity study (Ref. 26) have been used. Dose levels of 200, 600, and 2000 ppm for aniline hydrochloride in the diet were equivalent to dose levels of aniline of 7.2, 22 and 72 mg/kg/day. Tumors were observed in high dose males and one stromal sarcoma of the spleen was identified at 22 mg/kg/day. Based on these data the lowest dose of 7.2 mg/kg/day was used to define the No-Observed Effect Level for tumors (NOEL).

The PDE calculation is: (NOEL x body weight adjustment (kg)) / F1 x F2 x F3 x F4 x F5

The following safety factors as outlined in ICH Q3C have been applied to determine the PDE for aniline:
F1 = 5 (rat to human)
F2 = 10 (inter- individual variability)
F3 = 1 (study duration at least half lifetime)
F4 = 10 (severe toxicity – non-genotoxic carcinogenicity)

F5 = 1 (using a NOEL)

Lifetime PDE = 7.2 mg/kg/day x 50 kg / (5 x 10 x 1 x 10 x 1)

Lifetime PDE = 720 µg/day

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Benzyl Chloride (α-Chlorotoluene, CAS# 100-44-7)

Potential for human exposure

Human exposure is mainly occupational *via* inhalation while less frequent is exposure from ingesting contaminated ground water.

Mutagenicity/genotoxicity

Benzyl chloride is mutagenic and genotoxic *in vitro* but not in mammalian systems *in vivo*.

The International Agency for Research on Cancer (IARC) published a monograph performing a thorough review of the mutagenicity/genotoxicity data for benzyl chloride (Ref. 1). Some of the key conclusions are summarized here.

Benzyl chloride is mutagenic in:
Microbial reverse mutation assay (Ames) in *Salmonella typhimurium* strain TA100. Results of the standard assay are inconsistent across and within laboratories, but clear increases are obtained when testing in the gaseous phase (Ref. 2);
Chinese hamster cells (Ref. 1).

Benzyl chloride did not induce micronuclei *in vivo* in mouse bone marrow following oral, intraperitoneal or subcutaneous administration, but did form DNA adducts in mice after i.v. administration (Ref. 1).

Carcinogenicity

Benzyl chloride is classified as Group 2A, probably carcinogenic to humans (Ref. 3).

Benzyl chloride was administered in corn oil by gavage 3 times/week for 104 weeks to F-344 rats and B6C3F1 mice (Ref. 4). Rats received doses of 0, 15, or 30 mg/kg (estimated daily dose: 0, 6.4, 12.85 mg/kg); mice received doses of 0, 50, or 100 mg/kg (estimated daily dose: 0, 21.4, 42.85 mg/kg). In rats, the only statistically significant increase in the tumor incidence was for thyroid C-cell adenoma/carcinoma in the female high-dose group (27% versus 8% for control). A discussion of whether these thyroid tumors were treatment-related is included below. Several toxicity studies were conducted but C-cell hyperplasia was noted only in this lifetime study and only in female rats.

In mice (Ref. 4), there were statistically significant increases in the incidence of forestomach papillomas and carcinomas (largely papillomas) at the high dose in both males and females (62% and 37%, respectively, compared with 0% in controls). Epithelial hyperplasia was observed in the stomachs of animals without tumors. There were also statistically significant increases in male but not female mice in hemangioma or hemangiosarcoma (10% versus 0% in controls) at the high dose and in carcinoma or adenoma in the liver but only at the low dose (54% versus 33% in controls). In female, but not male, mice there were significant increases in the incidence of alveolar-bronchiolar adenoma or carcinoma at the high dose (12% versus 1.9% in controls).

Additional studies to assess carcinogenic potential were conducted but were not considered of adequate study design for use in calculating an AI. In one of three topical studies (Ref. 5) skin carcinomas were increased, although not statistically significantly (15% versus 0% in benzene controls). Initiation-promotion studies to determine the potential of benzyl chloride

to initiate skin cancer, using croton oil and the phorbol ester TPA (12-O-tetradecanoyl-phorbol-13-acetate) as promoters (Ref. 6, 7, 8) were of limited duration and the published reports were presented as preliminary findings, but no final results have been located in the literature. Injection site sarcomas were seen after subcutaneous administration (Ref. 9).

Benzyl chloride – Details of carcinogenicity studies

Study	**Animals/dose group**	**Duration/ Exposure**	**Controls**	**Doses**	**Most sensitive tumor site/type/sex or tumor observations**	**TD_{50} (mg/kg/d)**
Ref. 4*	52/sex/group F344 rat	2 year 3 times/wk Gavage	52	**2:** 15 and 30 mg/kg (6 and 12 mg/kg/d)	Thyroid C-cell neoplasm/ Female	40.6
Ref. 4	52/sex/group B6C3F1 mouse	2 year 3 times/wk Gavage	52	**2:** 50 and 100 mg/kg (21 and 42 mg/kg/d)	Forestomach papilloma, carcinoma/ Male	49.6
Ref. 5	11/group female ICR mouse	9.8 mo 3 times/wk for 4 wks, 2 times/wk Dermal	Yes (benzene treated)	**1:** 10 µL	No skin tumors	NC ^
Ref. 5	20/group female ICR mouse	50 weeks 2 times/wk Dermal	20 (benzene treated)	**1:** 2.3 µL	Skin squamous cell carcinoma	NC ^
Ref. 6	20/group male ICI Swiss albino mouse	>7 mo 2 times/wk Dermal, in toluene	20	**1:** 100 µg/mouse	No skin tumors	NC ^
Ref. 9	14 (40 mg/kg), and 8 (80 mg/kg) BD rat	51 weeks 1 time/wk Subcutaneous	Yes	**2:** 40 and 80 mg/kg/wk	Injection site sarcoma	NC ^
Ref. 7	40/sex/group Theiler's Original mouse	10 mo 1 dose (in toluene); wait 1 wk Promoter (croton oil)	40	**1:** 1 mg/ mouse	No skin tumors	NC ^

Study	Animals/dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/type/sex or tumor observations	TD_{50} (mg/kg/d)
		2 times/wk				
Ref. 8	Sencar mice	6 mo 1 dose; Promoter (TPA) 2 times/wk	Yes	3: 10; 100 and 1000 µg/ mouse	20% skin tumors [5% in TPA controls] (DMBA controls had skin tumors by 11 weeks)	NC ^

Studies listed are in CPDB (Ref. 10) unless otherwise noted.
*Carcinogenicity study selected for AI calculation.
^NC= Not calculated; small group size, limited duration. Not included in CPDB as route with greater likelihood of systemic exposure is considered more relevant.

Mode of action for carcinogenicity

The tumor types with the lowest calculated TD_{50} (highest potency) in the CPDB (Ref. 10) for benzyl chloride are forestomach tumors in mice and thyroid C-cell tumors in female rats. The relevance of the forestomach tumors to human risk assessment for low, non-irritating doses such as those associated with a potential impurity is highly questionable.

Forestomach tumors in rodents have been the subject of much discussion in assessment of risk to humans. With non-mutagenic chemicals, it is recognized that after oral gavage administration, inflammation and irritation related to high concentrations of test materials in contact with the forestomach can lead to hyperplasia and ultimately tumors. Material introduced by gavage can remain for some time in the rodent forestomach before discharge to the glandular stomach, in contrast to the rapid passage through the human esophagus. Such tumor induction is not relevant to humans at non-irritating doses. The same inflammatory and hyperplastic effects are also seen with mutagenic chemicals, where it is more complex to determine relative contribution to mode of action of these non-mutagenic, high-dose effects compared with direct mutation induction. However, often a strong case can be made for site-of-contact tumorigenesis that is only relevant at concentrations that cause irritation/inflammation, potentially with secondary mechanisms of damage. Cell proliferation is expected to play an important role in tumor development such that there is a non-linear dose response and the forestomach (or other site-of-contact) tumors are not relevant to low-dose human exposure.

Proctor *et al* (Ref. 11) proposed a systematic approach to evaluating relevance of forestomach tumors in cancer risk assessment, taking into account whether any known genotoxicity is potentially relevant to human tissues (this would include whether a compound is genotoxic *in vivo*), whether tumors after oral administration of any type are specific to forestomach, and whether tumors are observed only at doses that irritate the forestomach or exceed the MTD.

As described above and in the table, benzyl chloride predominantly induces tumors at the site-of-contact in rats and mice following exposure to high doses by gavage (forestomach tumors), by injection (injection site sarcoma) and by topical application in a skin tumor initiation-promotion model in sensitive Sencar mice. An OECD report in the Screening Information Dataset (SIDS) for high volume chemicals describes benzyl chloride as intensely irritating to skin, eyes, and mucous membranes in acute and repeat dose studies (Ref. 12). Groups of 10 Fischer 344 rats of both sexes died within 2-3 weeks from severe acute and chronic gastritis of the forestomach, often with ulcers, following oral administration 3 times/week of doses $\geq$ 250 mg/kg for males and $\geq$125 mg/kg for females (Ref. 4). Proliferative changes observed in female rats at lower doses included hyperplasia of the forestomach (62 mg/kg), and hyperkeratosis of the forestomach (30 mg/kg). The incidence of forestomach tumors was high in mice in the carcinogenicity study, and Lijinsky *et al* (Ref. 4) also observed non-neoplastic lesions in the forestomach of the rat in the subchronic range-finding study, but few forestomach neoplasms developed in the rat carcinogenicity assay. Due to the steepness of the dose-response curve and the difficulty establishing the MTD for rats, the author speculates that it was possible that the dose used in the rat study was marginally too low to induce a significant carcinogenic effect in rats.

In the case of benzyl chloride, other tumor types were discussed as possibly treatment-related besides those at the site-of-contact. In the mouse oral bioassay, Lijinsky characterized the carcinogenic effects other than forestomach tumors as *"marginal",* comprising an increase of endothelial neoplasms in males, alveolar-bronchiolar neoplasms of the lungs only in female mice (neither of these is statistically significant) and hepatocellular neoplasms only in low dose male mice (this tumor type was discounted as not dose related). It is of note that OECD SIDS (Ref. 12) reports observations of severe to moderate dose-related liver hyperplasia in a 26-week oral toxicity study in mice.

Statistically significant increases were reported in hemangiomas/hemangiosarcomas of the circulatory system in the male mice (TD_{50} 454 mg/kg/day), and in thyroid C-cell adenomas or carcinomas in the female rats (TD_{50} 40.6 mg/kg/day). The levels of thyroid C-cell tumors in female rats in the high dose group, while higher than female concurrent controls, (14/52 versus 4/52 in controls) were similar to the levels in the male concurrent controls (12/52). In males, thyroid C-cell tumor levels were lower in treated than in control rats. In a compilation of historical control data from Fisher 344 rats in the NTP studies (Ref. 13, 14), males and females show comparable levels of C-cell adenomas plus carcinomas in this rat strain, although the range is wider in males. Thus it is likely justifiable to compare the thyroid tumor levels in female rats treated with benzyl chloride with the concurrent controls of both sexes, and question whether the female thyroid tumors are treatment-related, although they were higher than the historical control range cited at the time (10%).

Regulatory and/or published limits

The US EPA (Ref. 15) derived an Oral Slope Factor of 1.7×10^{-1} per (mg/kg)/day, which corresponds to a 1 in 100,000 risk level of 2 µg/L or approximately 4 µg/day using US EPA assumptions.

Acceptable intake (AI)

Rationale for selection of study for AI calculation

The most robust evaluation of the carcinogenic potential of benzyl chloride was the Lijinsky *et al* study (Ref. 4) that utilized oral (gavage) administration. In this study, the animals were treated 3 days a week rather than 5 days a week as in a typical NCI/NTP study. Overall, however, the rat study is considered adequate for calculation of an AI because there was evidence that the top dose was near the maximum tolerated dose. In a 26-week range finding study described in the same report (Ref. 4), all ten rats of each sex given 125 or 250 mg/kg (3 days per week) died within 2-3 weeks. The cause of death was severe gastritis and ulcers in the forestomach; in many cases there was also myocardial necrosis. At 62 mg/kg, only 4 of 26 females survived to 26 weeks, and myocardial necrosis and forestomach hyperplasia were seen; hyperkeratosis of the forestomach was seen in some females at 30 mg/kg. At 62 mg/kg benzyl chloride, there was a decrease in body weight gain in both sexes, which was statistically significant in males. Thus, the high dose chosen for the carcinogenicity study was 30 mg/kg (3 times per week). At this dose, there was no difference from controls in survival in the 2-year carcinogenicity study, but 3 male rats had squamous cell carcinomas and papillomas of the forestomach, so it is unlikely that a lifetime study could have been conducted at a higher dose.

As described in the Methods Section 2.2, linear extrapolation from the TD_{50} was used to derive the AI. As described above, it is highly unlikely that benzyl chloride poses a risk of site-of-contact tumors in humans exposed to low concentrations as impurities in pharmaceuticals, well below concentrations that could cause irritation/inflammation. Therefore, the observed forestomach tumors in male mice are not considered relevant for the AI calculation. The significance of the thyroid C-cell tumors in female rats is also questionable since these tumors occur commonly in control rats. However, given the uncertain origin of these tumors, the thyroid C-cell tumors were used to derive the AI since they were associated with the lowest TD_{50}: 40.6 mg/kg/day.

Calculation of AI

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 40.6 (mg/kg/day)/50,000 x 50 kg

Lifetime AI = 40.6 µg/day (41 µg/day)

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Bis(chloromethyl)ether (BCME, CAS# 542-88-1)

Potential for human exposure

Industrial use, mainly via inhalation with minimal environmental exposure as result of rapid degradation in the environment, which is supported by the reported absence of BCME in ambient air or water (Ref. 1).

Mutagenicity/genotoxicity

BCME is mutagenic and genotoxic *in vitro* and *in vivo*.

BCME is mutagenic in:
Microbial reverse mutation assay (Ames), *Salmonella typhimurium* (Ref. 2).

In vivo, BCME did not cause chromosomal aberrations in bone-marrow cells of rats exposed by inhalation for six months (Ref. 3). A slight increase in the incidence of chromosomal aberrations was observed in peripheral lymphocytes of workers exposed to BCME (Ref. 4).

Carcinogenicity

BCME is classified by US EPA as a Group A, known human carcinogen (Ref. 5), and by IARC as a Group 1 compound, carcinogenic to humans (Ref. 6).

As described in the above reviews, numerous epidemiological studies have demonstrated that workers exposed to BCME (*via* inhalation) have an increased risk for lung cancer. Following exposure by inhalation, BCME is carcinogenic to the respiratory tract of rats and mice as described in the following studies:

The study of Leong *et al* (Ref. 3) was selected for derivation of the AI based on the most robust study design and the lowest TD_{50} value. Groups of male Sprague-Dawley rats and Ha/ICR mice were exposed by inhalation to 1, 10, and 100 ppb of BCME 6 h/day, 5 days/week for 6 months and subsequently observed for the duration of their natural lifespan (about 2 years). Evaluation of groups of rats sacrificed at the end of the 6-month exposure period revealed no abnormalities in hematology, exfoliative cytology of lung washes, or cytogenetic parameters of bone marrow cells. However, 86.5% of the surviving rats which had been exposed to 100 ppb (7780 ng/kg/day, or ~8 µg/kg/day) of BCME subsequently developed nasal tumors (esthesioneuroepitheliomas, tumors of the olfactory epithelium, which are similar to the rare human neuroblastoma) and approximately 4% of the rats developed pulmonary adenomas. Tumors were not observed in rats exposed to 10 or 1 ppb of BCME. Mice exposed to 100 ppb of BCME did not develop nasal tumors, but showed a significant increase in incidence of pulmonary adenomas over the control mice. Mice exposed to 10 or 1 ppb of BCME did not show a significant increase in incidence of pulmonary adenomas.

In an inhalation study, male Sprague-Dawley rats were exposed to BCME at a single dose level of 0.1 ppm (100 ppb) 6 h/day, 5 days/week for 10, 20, 40, 60, 80, or 100 days, then observed for the remainder of their lifetimes (Ref. 7). There was a marked increase in the incidence of several types of respiratory tract tumors in the treated animals compared with the controls.

BCME is a site-of-contact carcinogen, producing injection site sarcomas (Ref. 8) and skin tumors in mice, (Ref. 9); it also induces lung adenomas in newborn mice following sub-cutaneous application (Ref. 10).

Bis(chloromethyl)ether (BCME) – Details of carcinogenicity studies

Study	Animals/dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/type/sex	TD_{50} (mg/kg/d)
Ref. 3*	~104/group Rat, male Sprague-Dawley.	28 weeks 6 h/d, 5 d/wk Inhalation	104	**3:** 1; 10; 100 ppb (53;528; 7780 ng/ kg/d)	Nasal passage - esthesioneuro-epitheliomas	0.00357
Ref. 3	138-144/ group Mouse, male ICR/Ha.	25 weeks 6 h/d, 5 d/wk Inhalation	157	**3:** 1; 10; 100 ppb (0.295; 2.95;33.6 ng/kg/d)	Lung adenomas	No significant increases
Ref. 7	30-50 treated for different durations with same concentration, male Sprague Dawley rats.	6h/d, 5d/wk, for 10, 20, 40, 60, 80, and 100 exposures. Inhalation	240	**1:** 0.1 ppm	Lung and nasal cancer	NC^
Ref. 7	100/group male Golden Syrian Hamsters.	Lifetime 6h/d, 5d/wk, Inhalation	NA	**1:** 1 ppm	One undifferentiated in the lung	NC^
Ref. 9	50/group female ICR/Ha Swiss mice.	424-456 days, once weekly Intra-peritoneal	50	**1:** 0.114 mg/kg/d	Sarcoma (at the injection site)	0.182

Studies listed are in CPDB (Ref. 11) unless otherwise noted.
*Carcinogenicity study selected for AI calculation
^NC= Not calculated due to non-standard carcinogenicity design. Not in CPDB.
NA= Not available since controls were not reported in the study

Mode of action for carcinogenicity

BCME is a mutagenic carcinogen, and the acceptable intake is calculated by linear extrapolation from the TD50.

Regulatory and/or published limits

The US EPA (Ref. 5), calculated an oral cancer slope factor of 220 per mg/kg/day based on linearised multistage modelling of the inhalation study data by Kuschner *et al* (Ref. 7). The inhaled (and oral) dose associated with a 1 in 100,000 lifetime cancer risk is 3.2 ng/day (1.6 x 10^{-8} mg/m^3 for inhalation, 1.6 x 10^{-6} mg/L for oral exposure).

Acceptable intake (AI)

Rationale for selection of study for AI calculation

BCME is an *in vitro* mutagen, causes cancer in animals and humans and is classified as a known human carcinogen. Oral carcinogenicity studies were not conducted, so that intraperitoneal injection and inhalation studies are considered as a basis for setting an AI. The most sensitive endpoint was an increase in nasal tumors (esthesioneuroepitheliomas) in male rats in the inhalation carcinogenicity study (Ref. 3), with a TD_{50} of 3.57µg/kg/day. The AI derived by linear extrapolation from that TD_{50}, ~4ng/day, is essentially the same as the 3.2 ng/day recommendation of the US EPA. The study (Ref. 3) had a reliable design with multiple dose levels and >50 animals per dose group.

Evidence for tumors at other sites than those exposed by inhalation is lacking; the study cited above (Ref. 10) that describes lung tumors in newborn mice following skin application may not be definitive if inhalation may have occurred as a result of skin application. However, the AI derived here from inhalation data is considered applicable to other routes, because it is highly conservative (orders of magnitude below the default TTC of 1.5 µg/day). The AI is also similar to the limit derived by US EPA (based on inhalation data) that is recommended both for inhalation and ingestion (drinking water) of BCME (4 ng/day *vs* 3.2 ng/day).

Calculation of AI

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 3.57 µg/kg/day/50,000 x 50

Lifetime AI = 0.004 µg/day or 4 ng/day

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p-Chloroaniline (CAS# 106-47-8) and *p*-Chloroaniline HCl (CAS# 20265-96-7)

Potential for human exposure

Industrial exposure is primarily derived from the dye, textile, rubber and other industries (Ref. 1). If released into the environment, it is inherently biodegradable in water under aerobic conditions (Ref. 2).

Mutagenicity/Genotoxicity

p-Chloroaniline is mutagenic *in vitro*, with limited evidence for genotoxicity *in vivo*.

A detailed review of genotoxicity testing in a range of systems is provided by WHO (Ref. 3) with references, so only key conclusions are summarized here.

p-Chloroaniline is mutagenic in:
Microbial reverse mutation assay (Ames); 2 to 3-fold increase in revertants was seen in some laboratories but not in others.
Positive results reported in the mouse lymphoma L5178Y cell *tk* assay (Ref. 3) are small increases, associated with substantial cytotoxicity, and do not meet the current criteria for a positive assay using the "global evaluation factor" (Ref. 4).

Small increases in chromosomal aberrations in Chinese hamster ovary cells were not consistent between two laboratories.

In vivo, a single oral treatment did not induce micronuclei in mice at 180 mg/kg, but a significant increase was reported at 300 mg/kg/day after 3 daily doses in mice.

Carcinogenicity

p-Chloroaniline is classified by IARC as Group 2B, possibly carcinogenic to humans with adequate evidence of carcinogenicity in animals and inadequate evidence in humans (Ref. 5).

Carcinogenicity studies in animals have been conducted for *p*-chloroaniline or its hydrochloride salt, *p*-Chloroaniline HCl.

The NTP (Ref. 6) oral gavage study was used to calculate the AI, where *p*-chloroaniline HCl was carcinogenic in male rats, based on the increased incidence of spleen tumors: (Combined incidence of sarcomas: vehicle control, 0/49; low dose, 1/50; mid dose, 3/50; high dose, 38/50). Fibrosis of the spleen, a preneoplastic lesion that may progress to sarcomas, was seen in both sexes (Ref. 6, 7). In female rats, splenic neoplasms were seen only in one mid-dose rat and one high-dose rat. Increased incidences of pheochromocytoma of the adrenal gland in male and female rats may have been related to *p*-chloroaniline administration; malignant pheochromocytomas were not increased. In male mice, the incidence of hemangiosarcomas of the liver or spleen in high dose group was greater than that in the vehicle controls (4/50 in 0 mg/kg/day; 4/49 in 2.1 mg/kg/day; l/50 in 7.1 mg/kg/day; 10/50 in 21.4 mg/kg/day). The incidences of hepatocellular adenomas or carcinomas (combined) were increased in dosed male mice; of these, the numbers of hepatocellular carcinomas were (3/50 in 0 mg/kg/day; 7/49 in 2.1 mg/kg/day; 11/50 in 7.1 mg/kg/day; 17/50 in 21.4 mg/kg/day). The female mouse

study was negative. The final conclusion of NTP (Ref. 6) was that there was clear evidence of carcinogenicity in male rats, equivocal evidence of carcinogenicity in female rats, some evidence of carcinogenicity in male mice, and no evidence of carcinogenicity in female mice.

An earlier study used *p*-chloroaniline administered in feed to rats and mice (Ref. 8). Splenic neoplasms were found in dosed male rats and hemangiomatous tumors in mice. While the incidences of these tumors are strongly suggestive of carcinogenicity, NCI concluded that sufficient evidence was not found to establish the carcinogenicity of *p*-chloroaniline in rats or mice under the conditions of these studies. Since *p*-chloroaniline is unstable in feed, the animals may have received the chemical at less than the targeted concentration (Ref. 3). Therefore, this study is deemed inadequate.

***p*-Chloroaniline and *p*-Chloroaniline HCl – Details of carcinogenicity studies**

Study	**Animals/ dose group**	**Duration/ Exposure**	**Controls**	**Doses**	**Most sensitive tumor site/type/sex**	**TD_{50} (mg/kg/d)**
Ref. 6* *p*-chloroaniline HCl	50/group male B6C3F1 mice	103 weeks 5 times/ wk Gavage	50	**3:** 3; 10; 30 mg/kg (2.1; 7.1; 21.4 mg/kg/d)	Hepatocellular adenomas or carcinomas	33.8
Ref. 6 *p*-chloroaniline HCl	50/group female B6C3F1 mice	103 weeks 5 times/ wk Gavage	50	**3:** 3; 10; 30 mg/kg (2.1; 7.1; 21.4 mg/kg/d)	Negative	NA
Ref. 6 *p*-chloroaniline HCl	50/group male Fischer 344 rat	103 weeks 5 times/ wk Gavage	50	**3:** 2; 6;18 mg/kg (1.4; 4.2; 12.6 mg/kg/d)	Spleen fibrosarcoma, haemangiosarcoma, osteosarcoma	7.62
Ref. 6 *p*-chloroaniline HCl	50/group female Fischer 344 rat	103 weeks 5 times/ wk Gavage	50	**3:** 2; 6; 18 mg/kg (1.4; 4.2; 12.6 mg/kg/d)	No significant increases; equivocal	NA
Ref. 8	50/group male Fischer 344 rat	78 weeks (study duration: 102 wk) Diet	20	**2:** 250; 500 ppm (7.7; 15.2 mg/kg/d)	Mesenchymal tumors (fibroma, fibrosarcoma, haemangiosarcoma, osteosarcoma, sarcoma not otherwise specified)	72

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/type/sex	TD_{50} (mg/kg/d)
					of the spleen or splenic capsule	
Ref. 8	50/group female Fischer 344 rat	78 weeks (study duration: 102 wk) Diet	20	**2:** 250; 500 ppm (9.6, 19 mg/kg/d)	Negative	NA
Ref. 8	50/group male B6C3F1 mice	78 weeks (study duration: 91 wk) Diet	20	**2:** 2500; 5000 ppm (257;275 mg/kg/d)	Haemangiosarcomas (subcutaneous tissue, spleen, liver, kidney). Increased incidence of all vascular tumors	Not significant (CPDB)
Ref. 8	50/group female B6C3F1 mice	78 weeks (study duration: 102 wk) Diet	20	**2:** 2500; 5000 ppm (278, 558 mg/kg/d)	Haemangiosarcomas (liver and spleen). Increased incidence of combined vascular tumors	1480

Studies listed are in CPDB (Ref. 9)
*Carcinogenicity study selected for AI calculation.
NA = Not applicable

Mode of action for carcinogenicity

p-Chloroaniline induced tumors in male rats, such as spleen fibrosarcomas and osteosarcomas, typical for anline and related chemicals. Repeated exposure to *p*-chloroaniline leads to cyanosis and methemoglobinemia, followed by effects in blood, liver, spleen, and kidneys, manifested as changes in hematological parameters, splenomegaly, and moderate to severe hemosiderosis in spleen, liver, and kidney, partially accompanied by extramedullary hematopoiesis (Ref. 6, 8). These effects occur secondary to excessive compound-induced hemolysis and are consistent with a regenerative anemia (Ref. 3). The evidence supports an indirect mechanism for tumorigenesis, secondary to methemoglobinemia, splenic fibrosis and hyperplasia (Ref. 10), and not tumor induction related to a direct interaction of *p*-chloroaniline or its metabolites with DNA. Similarly, the reported induction of micronuclei *in vivo* is likely to be secondary to regenerative anemia/altered erythropoeisis, as with aniline (Ref. 11,12).

The tumor type with the lowest TD_{50} was spleen tumors in male rats. However, since this tumor type is associated with a non-linear dose relation, spleen tumors were not used to calculate the acceptable intake. Based on non-neoplastic (hematotoxic) effects, WHO (Ref. 3) recommends a level of 2 µg/kg/day, i.e., 100 µg/day for a 50 kg human.

Although the *in vitro* mutagenicity data for *p*-chloroaniline indicate small increases in mutations that are not reproducible across laboratories, a mutagenic component to a mode of action for liver tumors cannot be ruled out.

Regulatory and/or published limits

No regulatory limits have been published for *p*-chloroaniline or the hydrochloride salt.

Acceptable intake (AI)

Because a mutagenic component to the mode of action for male mouse liver tumors cannot be ruled out, the AI was derived by linear extrapolation from the TD_{50} of 33.8 mg/kg/day for combined numbers of adenomas and carcinomas.

Calculation of AI

Based on male mouse liver tumors for *p*-chloroaniline HCl

Lifetime AI = TD_{50}/50,000 x 50kg

Lifetime AI = 33.8mg/kg/day /50,000 x 50 kg

Lifetime AI = 34 µg/day

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1-Chloro-4-Nitrobenzene (para-Chloronitrobenzene, CAS# 100-00-5)

Potential for human exposure

Potential for exposure is in industrial use. No data are available for exposure of the general population.

Mutagenicity/genotoxicity

Chloro-4-nitrobenzene is mutagenic and genotoxic *in vitro* and *in vivo*.
Chloro-4-nitrobenzene was mutagenic in:
Microbial reverse mutation assay (Ames) *Salmonella typhimurium* strains TA100 and TA1535 in the presence of S9 metabolic activation, and was negative in TA1537, TA1538, TA98, and *E.coli* WP2*uvrA* (Ref. 1, 2, 3, 4). It was also weakly positive without metabolic activation in TA1535 in 2 of 4 studies (Ref. 4).

In vivo, DNA strand breaks were induced in the liver, kidney, and brain of male Swiss mice when chloro-4-nitrobenzene was administered intraperitoneally (Ref. 5, 6).

Carcinogenicity

1-Chloro-4-nitrobenzene is classified by IARC as a Group 2 carcinogen, not classifiable as to its carcinogenicity in humans (Ref. 7) and US EPA considers it to be a Group B2 carcinogen or probable human carcinogen (Ref. 8).

Animal carcinogenicity studies have been conducted with 1-chloro-4-nitrobenzene by administration in the feed to rats and mice (Ref. 9, 10) or by gavage in male rats (Ref. 12).

In a 2-year diet study (Ref. 9), there were significant increases in spleen tumors (fibroma, fibrosarcoma, osteosarcoma and sarcoma) in rats of both sexes, and there were increases in spleen hemangiosarcomas in both sexes, that were statistically significant in males at the mid and high doses (7.7 and 41.2 mg/kg/day). Non-neoplastic changes of the spleen such as fibrosis, and capsule hyperplasia were seen. An increase in adrenal medullary pheochromocytomas was seen at the high dose that was statistically significant in females (53.8 mg/kg/day). In mice, the only significant increase in tumors was in liver hemangiosarcomas at the high dose in females (275.2 mg/kg/day). Hematologic disturbances such as decreases in red blood cell numbers and haematocrit, and extramedullary hematopoiesis, were seen both in rats and in mice.

In another diet study (Ref. 10), 1-chloro-4-nitrobenzene did not induce tumors in male CD-1 rats when fed in the diet for 18 months. The concentration in the diet was adjusted during the 18-month period due to toxicity as follows: The low dose group received 2000 ppm for the first 3 months, 250 ppm for next 2 months, and 500 ppm from 6 to 18 months; the high dose group received 4000 ppm for the first 3 months, 500 ppm for next 2 months, and 1000 ppm from 6 to 18 months. The average daily exposure was approximately 17 and 33 mg/kg for the low and high dose groups, respectively. Rats were sacrificed 6 months after the last dose and examined for tumors. No treatment-related increases in tumors were observed in the 11 tissues examined (lung, liver, spleen, kidney, adrenal, heart, bladder, stomach, intestines, testes and pituitary).

The same laboratory (Ref. 10) also investigated the carcinogenic potential of 1-chloro-4-nitrobenzene in male and female CD-1 mice, given in the diet for 18 months. Mice were sacrificed 3 months after the last exposure and 12 tissues (lung, liver, spleen, kidney, adrenal, heart, bladder, stomach, intestines, and reproductive organs) were examined for tumors. A dose-dependent increase in vascular tumors (hemangiomas or hemangiosarcomas) of liver, lung, and spleen was observed in both male and female mice.

In an oral study (Ref. 11), male and female Sprague-Dawley rats (n = 60) were given 1-chloro-4-nitrobenzene by gavage 5 days/week for 24 months. In both sexes, toxicity was observed: methemoglobinemia in mid- and high-dose groups, and hemosiderin and anemia in the high-dose group.

1-Chloro-4-nitrobenzene – Details of carcinogenicity studies

Study	**Animals/ dose group**	**Duration/ Exposure**	**Controls**	**Doses**	**Most sensitive tumor site/type/sex**	**TD_{50} (mg/kg/d)**
Ref. 9*+	50/group male F344 rats (SPF)	2 years (Diet)	50	**3:** 40; 200; 1000 ppm. (1.5; 7.7; 41.2 mg/kg/d)	Spleen hemangiosarcomas 7.7 mg/kg/d	173.5
	50/group female F344 rats (SPF)	2 years (Diet)	50	**3:** 40; 200; 1000 ppm. (1.9; 9.8;53.8 mg/kg/d)	Pheochromo-cytoma/Female 53.8 mg/kg/d	116.9**
	50/group male Crj:BDF1 (SPF)	2 years (Diet)	50	**3:** 125;500; 2000 ppm. (15.3; 60.1;240 .1 mg/kg/d)	NA	
	50/group female Crj:BDF1 (SPF)	2 years (Diet)	50	**3:** 125;500; 2000 ppm. (17.6; 72.6; 275.2 mg/kg/d)	Hepatic hemangiosarcomas 275.2 mg/kg/d	1919.9
Ref. 10	14-15/ group male CD-1	18 mo Diet; sacrificed	16	**2:** Average 17 and	NA	Negative^

	rats	6 mo after last dose		33 mg/kg; (see text) (22.6 and 45.2 mg/kg/d)		
	14-20/sex group CD-1 mice	18 mo Diet; sacrificed 3 mo after last dose	15/sex	**2:** M: 341; 720. F: 351; 780 mg/kg/d	Vascular (hemangiomas/ hemangio-sarcomas)/Male	430^
Ref. 11+	60/sex/ group Sprague Dawley rat	24 mo 5 d/ wk, Gavage	Yes	**3:** 0.1; 0.7; 5 mg/kg/d	NA	Negative

Studies listed are in CPDB (Ref. 12) unless otherwise noted..
*Carcinogenicity study selected for AI/PDE calculation.
**TD50 calculated based on carcinogenicity data (see Note 1)
+Not in CPDB.
^ Histopathology limited to 11-12 tissues.
NA = Not applicable

Mode of action for carcinogenicity

1-Chloro-4-nitrobenzene is significantly metabolized by reduction to 4-chloroaniline (*p*-chloroaniline) in rats (Ref. 13), rabbits (Ref. 14) and humans (Ref. 15). *p*-Chloroaniline has been shown to produce hemangiosarcomas and spleen tumors in rats and mice, similar to 1-chloro-4-nitrobenzene (Ref. 16). Like aniline, an indirect mechanism for vascular tumorigenesis in liver and spleen was indicated, secondary to oxidative erythrocyte injury and splenic fibrosis and hyperplasia, both for 4-chloroaniline (Ref. 16) and 1-chloro-4-nitrobenzene (Ref. 17). Methemoglobinemia and associated toxicity is a notable effect of 1-chloro-4-nitrobenzene. A non-linear mechanism for tumor induction is supported by the fact that in the oral gavage study (Ref. 11), carried out at lower doses than the diet studies (Ref. 9, 10), methemoglobinemia and hemosiderin were seen but there was no increase in tumors.

The tumor type with the lowest TD_{50} was adrenal medullary pheochromocytomas in female rats (Ref. 9). This tumor type is common as a background tumor in F344 rats, especially males, and is seen after treatment with a number of chemicals, many of them non-mutagenic (Ref. 18). It has been proposed that these tumors are associated with various biochemical disturbances, and the mode of action for induction of pheochromocytomas by chemicals such as aniline and *p*-chloroaniline that are toxic to red blood cells may be secondary to uncoupling of oxidative phosphorylation (Ref. 18) or perhaps hypoxia.

Overall, there is substantial evidence for a non-mutagenic mode of action as follows:
The most notable types of tumors induced were those associated with methemoglobinemia, (spleen and vascular tumors);
Adrenal medullary pheochromocytomas may be associated with the same perturbations;

There is clearly a non-linear dose relation (based on no-effect doses and on the negative results of the lower-dose study (Ref. 11).

However, in mutagenicity studies in *Salmonella*, 1-chloro-4-nitrobenzene was mutagenic in *Salmonella* TA100 and TA1535 (but not TA98 and other strains). This may indicate a mutagenic component to the mode of action for tumor induction by 1-chloro-4-nitrobenzene, and the pattern of mutagenicity is different from its metabolite *p*-chloroaniline, which was not consistently detected as mutagenic across laboratories, and was reproducibly mutagenic only in *Salmonella* TA98 with rat liver S9 (Ref. 19) indicating differences in mutagenic metabolites or mechanism. *In vivo* genotoxicity data are lacking to help assess potential for a mutagenic mode of action.

Since 1-chloro-4-nitrobenzene is mutagenic, and a mutagenic mode of action cannot be ruled out, an AI calculation was performed.

Regulatory and/or published limits

No regulatory limits have been published, for example by US EPA, WHO, or Agency for Toxic Substances & Disease Registry (ATSDR).

Calculation of AI

The most sensitive TD_{50} is that for adrenal medullary pheochromocytomas in female rats (Ref. 9).

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 117 mg/kg/day /50,000 x 50 kg

Lifetime AI = 117 µg/day

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p-Cresidine (2-Methoxy-5-Methyl Aniline, CAS# 120-71-8)

Potential for human exposure

Potential for exposure is in industrial use. No data are available for exposure of the general population.

Mutagenicity/Genotoxicity

p-Cresidine is mutagenic/genotoxic *in vitro* with equivocal evidence for genotoxicity *in vivo.*

p-Cresidine is mutagenic in:
Several *Salmonella* strains in the presence of metabolic activation (Ref. 1, 2, 3).
Big Blue transgenic mouse model with the lamda cII gene; *p*-cresidine was administered a diet of 0.25 and 0.5%, comparable to the doses in the carcinogenicity study, for 180 days (Ref. 4).

In vivo, *p*-cresidine did not induce micronuclei in bone marrow of mice (Ref. 5. 6, 7), or in p53 heterozygous or nullizygous mice (Ref. 8). Increases in micronuclei in another study in p53 heterozygous mice may be secondary to methemobolinemia and regenerative anemia as with aniline and related compounds (Ref. 9).

DNA strand breaks were not observed using the alkaline elution method in several tissues including bladder (Ref. 6; 7) but DNA strand breaks assessed by the Comet assay were reported in bladder mucosa, but not other tissues, after oral treatment of mice with *p*-cresidine (Ref. 10).

Carcinogenicity

p-Cresidine is classified by IARC as a Group 2B carcinogen, or possibly carcinogenic in humans (Ref. 11).

There is only one set of carcinogenicity studies in the standard rodent model. In NTP studies (Ref. 5) *p*-cresidine induced tumors in lifetime studies in Fischer 344 rats and B6C3F1 mice, with *p*-cresidine administered in the feed. No carcinogenicity data are available for other routes of exposure.

p-Cresidine was administered in the feed, to groups of 50 male and 50 female animals of each species. There were also 50 control animals of each sex. The concentrations of *p*-cresidine were 0.5 or 1.0 percent in the diet, but in mice the concentrations administered were reduced after 21 weeks to 0.15 and 0.3 percent. The dose levels, converted to mg/kg/day in the CPDB (Ref. 12), were 198 and 368 mg/kg/day for male rats; 245 and 491 mg/kg/day for female rats; 260 and 552 mg/kg/day for male mice and 281 and 563 mg/kg/day for female mice.

All dosed animals, except for high dose male mice, were administered *p*-cresidine in the diet for 104 weeks and observed for an additional period of up to 2 weeks. All high dose male mice were dead by the end of week 92. Mortality rates were dose-related for both sexes of both species. That incidences of certain tumors were higher in low dose than in high dose groups was probably due to accelerated mortality in the high dose groups.

In dosed rats of both sexes, statistically significant incidences of bladder carcinomas (combined incidences of papillary carcinomas, squamous-cell carcinomas, transitional-cell papillomas, transitional-cell carcinomas, and undifferentiated carcinomas) and olfactory neuroblastomas were observed. The combined incidence of neoplastic nodules of the liver, hepatocellular carcinomas, or mixed hepato/cholangio carcinomas was also significant in low dose male rats. In both male and female dosed mice, the incidence of bladder carcinomas (combined incidence of carcinomas, squamous-cell carcinomas, and transitional-cell carcinomas) was significant. The incidence of hepatocellular carcinomas was significant in dosed female mice.

In summary, *p*-cresidine was carcinogenic to Fischer 344 rats, causing increased incidences of carcinomas and of papillomas of the urinary bladder in both sexes, increased incidences of olfactory neuroblastomas in both sexes, and of liver tumors in males. *p*-Cresidine was also carcinogenic in B6C3F1 mice, causing carcinomas of the urinary bladders in both sexes and hepatocellular carcinomas in females.

Induction of bladder tumors was also seen in a short-term carcinogenicity model in p53+/- hemizygous mice. p-Cresidine was used as a positive control in a large inter-laboratory assessment of the mouse model (Ref. 13). Increases in bladder tumors were seen in 18 of 19 studies in which p-cresidine was administered by gavage at 400 mg/kg/day for 26 weeks, and in the single study where compound was given in feed.

***p*-Cresidine – Details of carcinogenicity studies**

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/type/ sex	TD_{50} (mg/kg/d)
Ref. 5*	50/sex/ group B6C3F1 mice	2 year Feed	50	**2:** 0.5 and 1% Reduced after 21 wk to 0.15 and 0.3%. M: 260:552. F: 281; 563 mg/kg/d	Urinary bladder /Male	44.7
Ref. 5	50/sex/ group Fisher 344 rats	2 year Feed	50	0.5 and 1% M: 198;396. F: 245;491 mg/kg/d	Urinary bladder /Male	88.4

*Carcinogenicity study selected for AI calculation.
Studies listed are in CPDB (Ref. 12).

Mode of action for carcinogenicity

p-cresidine is a mutagenic carcinogen, and the acceptable intake is calculated by linear extrapolation from the TD50.

Regulatory and/or published limits

No regulatory limits have been published

Acceptable intake (AI)

Rationale for selection of study for AI calculation**:**

The only adequate carcinogenicity studies of *p*-cresidine were those reported in the CPDB and conducted by NCI/NTP (Ref. 5). The study in mice was selected for derivation of the AI since the most sensitive TD_{50} was based on urinary bladder tumors in male mice.

Calculation of AI

The most sensitive TD_{50} values from the NCI/NTP studies are for the urinary bladder in both sexes of rats and mice; in rats the TD_{50} was 110 mg/kg/day for females and 88.4 mg/kg/day for males; in mice the TD_{50} was 69 mg/kg/day for females and 44.7 mg/kg/day for males. The most conservative value is that identified for male mice.

The lifetime AI is calculated as follows:

Lifetime AI = TD_{50}/50,000 x 50 kg
Lifetime AI = 44.7 mg/kg/day /50,000 x 50 kg

Lifetime AI = 45 µg/day

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Dimethylcarbamyl Chloride (CAS# 79-44-7)

Potential for human exposure

Potential for exposure is in industrial use. No data are available for exposure of the general population.

Mutagenicity/genotoxicity

Dimethylcarbamyl chloride (DMCC) is considered mutagenic and genotoxic *in vitro* and *in vivo*.

DMCC was mutagenic in:
Salmonella typhimurium TA100, TA1535, TA1537, TA98 and TA1538 with and without metabolic activation (Ref. 1, 2);

In vivo, positive results were seen in the micronucleus assay (Ref. 3).

Carcinogenicity

DMCC is classified by IARC as a Group 2A compound, or probably carcinogenic to humans (Ref. 4).

No deaths from cancer were reported in a small study of workers exposed for periods ranging from 6 months to 12 years, and there is inadequate evidence in humans for the carcinogenicity of DMCC. There is evidence that DMCC induced tumors in rodents.

Since oral studies are lacking, the studies considered for AI derivation used inhalation and intraperitoneal administration.

Syrian golden hamsters were exposed to 1 ppm DMCC by inhalation for 6 hours/day, 5 days/week until the end of their lives or sacrifice due to moribundity (Ref. 5). Squamous cell carcinoma of the nasal cavity was seen in 55% of the animals whereas no spontaneous nasal tumors were seen in the controls or historical controls. When early mortality was taken into consideration, the percentage of tumor bearing animals was calculated to be 75% (Ref. 5).

DMCC was tested for carcinogenic activity in female ICR/Ha Swiss mice by skin application, subcutaneous injection and intraperitoneal (i.p.) injection (Ref. 6; this study was selected to calculate the AI). In the skin application, 2 mg of DMCC was applied 3 times a week for 492 days; this was seen to induce papillomas in 40/50 mice and carcinomas in 30/50 mice. Subcutaneous injection once weekly was continued for 427 days at a dose of 5 mg/week. Sarcomas and squamous cell carcinomas were seen in 36/50 and 3/50 mice, respectively, after the subcutaneous injection. In the i.p. experiment, the mice were injected weekly with 1 mg DMCC for a total duration of 450 days. The treatment induced papillary tumors of the lung in 14/30 animals and local malignant tumors in 9/30 animals (8/30 were sarcomas). In the control groups, no tumors were seen by skin application, 1/50 sarcoma by subcutaneous injection, and 1/30 sarcoma and 10/30 papillary tumors of lung by i.p. injection. Overall, only the local (injection site) tumors were significantly increased; tumors at distant sites were not statistically significantly increased compared with controls.

Dimethylcarbamyl chloride – Details of carcinogenicity studies

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Tumor observations	TD_{50} (mg/kg/d)
Ref. 6*	30 female ICR/Ha Swiss mice	64 weeks Once/wk Intra-peritoneal	30	**1:** 1 mg 5.71 mg/kg/d	Injection site: malignant tumors/Female	4.59 ^^^
Ref. 5**	99 male Syrian golden hamsters	Lifetime 6 h/d, 5 d/wk Inhalation	50 sham treated 200 untreated	**1:** 1 ppm 0.553 mg/kg/d	Squamous cell carcinoma of nasal cavity	0.625
Ref. 6	50 female ICR/Ha Swiss mice	70 weeks 3 times/wk Skin	50	**1:** 2 mg	Skin: Papillomas and carcinomas/ Female	NA^
Ref. 6	50 female ICR/Ha Swiss mice	61 weeks Once/wk Subcutaneous	50	**1:** 5 mg	Injection site: Fibrosarcomas; Squamous cell carcinomas/ Female	NA^
Ref. 7	Male Sprague-Dawley rats	6 weeks 6 h/d, 5 d/wk Inhalation; examined at end of life	Yes	**1:** 1 ppm	Nasal tumors/Male	NA^^^^
Ref. 8	30-50 female ICR/Ha Swiss mice	18-22 mo 3 times/wk Skin	Yes	**2:** 2 and 4.3 mg	Skin. Mainly skin squamous carcinoma/Female	NA^
Ref. 8	Female ICR/Ha Swiss mice	18-22 mo Once/wk Subcutaneous	Yes	**1:** 4.3 mg	Site of administration. Mainly sarcoma. Hemangioma, squamous carcinoma and papilloma also seen/Female	NA^^
Ref. 8	Female ICR/Ha Swiss mice	12 mo Once/wk Subcutaneous; examined at end of life	Yes	**2:** 0.43 and 4.3 mg		NA^^

Studies listed are in CPDB (Ref. 9) unless otherwise noted.
*Carcinogenicity study selected for non-inhalation AI.
**Carcinogenicity study selected for inhalation AI.
NA= Not applicable

^Did not examine all tissues histologically. Subcutaneous and skin painting studies are not included in CPDB as route with greater likelihood of whole body exposure is considered more valuable.
^^Subcutaneous and skin painting studies are not included in CPDB as route with greater likelihood of whole body exposure is considered more valuable.
^^^Histopathology only on tissues that appeared abnormal at autopsy.
^^^^Examined only for nasal cancer. Does not meet criteria for inclusion in CPDB of exposure for at least one fourth of the standard lifetime.

Regulatory and/or published limits

No regulatory limits have been published.

Acceptable intake (AI)

Based on the above data, DMCC is considered to be a mutagenic carcinogen. As a result, linear extrapolation from the most sensitive TD_{50} in carcinogenicity studies is an appropriate method with which to derive an acceptable risk dose. Since DMCC appears to be a site-of-contact carcinogen, it was appropriate to derive a separate AI for inhalation exposure compared with other routes of exposure.

No information from oral administration is available, so that for routes of exposure other than inhalation, the study by Van Duuren *et al* (Ref. 6), with administration by i.p. injection, was used. The TD_{50} was 4.59 mg/kg/day based on mixed tumor incidences (CPDB).

The lifetime AI is calculated as follows:

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 4.59 mg/kg/day /50,000 x 50 kg

Lifetime AI = 5 µg/day

Inhalation AI

The inhalation AI is calculated as follows:

After inhalation of DMCC, nasal cancer in hamsters is the most sensitive endpoint and the TD_{50} was 0.625 mg/kg/day.

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 0.625 mg/kg/day /50,000 x 50 kg

Lifetime inhalation AI = 0.6 µg/day

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Dimethyl Sulfate (CAS# 77-78-1)

Potential for human exposure

Dimethyl sulfate (DMS) is found in ambient air with mean concentration of 7.4 µg per cubic meter or 1.4 ppb based on 1983 data compiled from a single site by the US EPA (Ref. 1).

Mutagenicity/genotoxicity

DMS is mutagenic/genotoxic *in vitro* and *in vivo* (Ref. 2).

DMS is mutagenic in:
The microbial reverse mutation assay (Ames), *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 with and without activation (Ref. 3).

In vivo, DMS forms alkylated DNA bases and is consistently positive in genotoxicity assays (Ref. 4). Elevated levels of chromosomal aberrations have been observed in circulating lymphocytes of workers exposed to DMS (Ref. 4).

Carcinogenicity

DMS is classified by IARC as a Group 2A carcinogen, probably carcinogenic to humans (Ref. 4).

No epidemiological studies were available for DMS although a small number of cases of human exposure and bronchial carcinoma have been reported. DMS is carcinogenic in animals by chronic and subchronic inhalation, and single and multiple subcutaneous injections; however, DMS has not been tested by the oral route of exposure. DMS is carcinogenic in rats, mice, and hamsters (Ref. 4). The carcinogenicity studies for DMS were limited for a variety of reasons and this is likely why DMS is not listed on the Carcinogenicity Potency Database (CPDB). The studies evaluating carcinogenicity of DMS are described below (excerpted from US EPA, Ref. 5).

DMS- Details of carcinogenicity studies

Study	Animals	Duration/ Exposure	Controls	Doses	Tumor observations	TD_{50} (mg/kg/d)
Ref. 6	Golden hamsters, Wistar rats, and NMRI mice male and female (number not clearly specified)	15 mo 6 h/d, 2 d/wk followed by 15 mo observation period Inhalation	Yes	**2:** 0.5; 2.0 ppm	Tumors in lungs, thorax and nasal passages at both doses	NA^
Ref. 7	20-27 BD rats Sex not specified	130 days 1 h/d, 5 d/wk followed by 643 day observation period Inhalation	No	**2:** 3; 10 ppm	Squamous cell carcinoma in nasal epithelium at 3 ppm. Squamous cell carcinomas in nasal epithelium and lympho-sarcoma in the thorax with metastases to the lung at 10 ppm.	NA^^
Ref. 8	8-17 BD Rats Sex not specified	394 days The duration of the study was not reported but mean tumor induction time was 500 days Subcutaneous	No	**2:** 8; 16 mg/kg/wk	Injection-site sarcomas in 7/11 at low dose and 4/6 at high dose; occasional metastases to the lung. One hepatic carcinoma.	NA^^^
Ref. 7	15 BD Rats Sex not specified	Up to 740 day evaluation Following single injection Subcutaneous	No	**1:** 50 mg/kg	Local sarcomas of connective tissue in 7/15 rats; multiple metastases to the lungs in three cases	NA^^^
Ref. 7	12 BD rats Sex not specified	800 days Once/wk Intravenous	No	**2:** 2; 4 mg/kg	No tumors reported	NA^^^

Study	Animals	Duration/ Exposure	Controls	Doses	Tumor observations	TD_{50} (mg/kg/d)
Ref. 7	8 BD rats (pregnant females)	1 year offspring observation following single dose, gestation day 15 Intravenous	No	**1:** 20 mg/kg	4/59 offspring had malignant tumors of the nervous system while 2/59 had malignant hepatic tumors.	NA^^^^
Ref. 9	90 female CBAX57 Bl/6 mice	Duration not reported 4 h/d, 5 d/wk Inhalation	Not indicated	3: 0.4; 1; 20 mg/m^3	Increase in lung adenomas at high dose	NA*
Ref. 10	20 ICR/Ha Swiss mice¥	475 days 3 times/wk Dermal	Not indicated	1: 0.1 mg	No findings	NA**

Studies listed are in not in CPDB.
NA = Not applicable
^ Control data not reported. Tumor incidences not tabulated by species or dose.
^^ Small group size. No concurrent control group. One rat at high dose had a cerebellar tumor and two at low dose had nervous system tumors which are very rare and distant from exposure.
^^^ Small group size, no concurrent control group.
^^^^ No concurrent control group.
* Duration not reported
** Limited number of animals. Only one dose tested. Even when DMS was combined with tumor promoters no tumors were noted.
¥ Sex not specified

Mode of action for carcinogenicity

Dimethyl Sulfate is a mutagenic carcinogen, and the acceptable intake is calculated by linear extrapolation from the TD50.

Regulatory and/or published limits

The European Union (EU) Institute for Health and Consumer Protection (ECHA, Ref.11) developed a carcinogenicity slope curve based on the inhalation carcinogenicity data for DMS. ECHA calculated a T_{25} (dose that resulted in a 25% increase in tumors) using the rat inhalation study (Ref. 7). Systemic effects (nervous system) and local nasal tumors were observed in this limited carcinogenicity study. However, as with other studies listed, this study was severely limited with high mortality, no control animals, only 2 dose groups and minimal pathological evaluations; therefore, the study was not suitable for linear extrapolation.

Acceptable intake (AI)

While DMS is considered to be a likely oral carcinogen and probable human carcinogen, there are no oral carcinogenicity studies from which to derive a TD_{50} value. Moreover, the inhalation studies that are available are limited for a variety of reasons and are not suitable for TD_{50} extrapolation. Given this, it is reasonable to limit DMS to the threshold of toxicological concern (TTC) lifetime level of 1.5 µg/day.

Lifetime AI = 1.5 µg/day

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Ethyl Chloride (Chloroethane, CAS# 75-00-3)

Potential for human exposure

Low levels (parts-per-trillion) from contaminated ambient air and drinking water. Dermal contact as a topical anesthetic.

Mutagenicity/genotoxicity

Ethyl chloride is mutagenic and genotoxic *in vitro* but not *in vivo*. IARC (Ref. 1) has reviewed the mutagenicity data for ethyl chloride; key points are summarized here.

Ethyl chloride was mutagenic in:
Microbial reverse mutation assay (Ames), *Salmonella typhimurium* strains TA100 and TA1535 and in *Escherichia coli* WP2*uvrA* with and without metabolic activation when tested in conditions that enable exposure to gas (Ref. 2, 3, 4);
CHO cell *hprt* assay with and without metabolic activation.

In vivo ethyl chloride was negative in a mouse bone marrow micronucleus test after inhalation at approximately 25,000 ppm for 3 days, and in an Unscheduled DNA Synthesis (UDS) assay in female mouse liver (Ref. 5).

Carcinogenicity

Ethyl chloride was designated by IARC as Class 3, or not classifiable as to its carcinogenicity (Ref. 1).

Only one carcinogenicity study was found for ethyl chloride, NTP studies (Ref. 6) in rats and mice of both sexes *via* inhalation for 6 h/day, 5 days/week for 100 weeks. The single exposure concentration (15,000 ppm) tested was limited by safety concern (explosion risk) and on the lack of obvious effect in a 3 month range-finding study up to 19,000 ppm. These data were later assessed by US EPA (Ref. 7), comparing ethyl chloride with ethyl bromide. Ethyl chloride was notable because, along with structurally similar ethyl bromide, it induced very high numbers of uncommon uterine tumors (endometrial carcinomas) in mice, but not rats. Ethyl chloride produced clear evidence of carcinogenicity in female mice (uterus) and equivocal evidence of carcinogenicity in male and female rats. Due to poor survival, the male mouse study was considered inadequate although there was an increased incidence of lung tumors.

Ethyl Chloride – Details of carcinogenicity studies

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/sex	TD_{50} (mg/kg/d)
Ref. 6, 7*	50/sex/ group B6C3F1 mice	100 weeks 6 h/d, 5 d/wk Inhalation	50	**1:** M: 10.4 F: 12.4 g/kg/d	Uterus/Female	1810

Ref. 6, 7	50/sex/ group Fischer 344 rats	100 weeks 6 h/d, 5 d/wk Inhalation	50	**1:** M: 2.01 F: 2.88 g/kg/d	Negative	NA

*Carcinogenicity study selected for AI calculation. Studies listed are in CPDB (Ref. 8).
NA = Not applicable

Mode of action of carcinogenicity

Holder (Ref. 7) proposes reactive metabolites may contribute to carcinogenicity, but notes female mice have a marked stress response to ethyl chloride exposure at the high concentrations used in the carcinogenicity study; such stress has been shown to lead to adrenal stimulation. It was proposed that high corticosteroid production could promote development of endometrial cancers in mice.

Regulatory and/or published limits

The US EPA established an inhalation Reference Concentration (RfC) for non-carcinogenic effects of 10 mg/m^3, or 288 mg/day assuming a respiratory volume of 28,800 L/day (Ref. 9).

Acceptable intake (AI)

Rationale for selection of study for AI calculation

Although the studies are not robust in design (having a single dose group), the high level of a specific rare type of uterine carcinoma of endometrial original in mice (43/50 affected compared with 0/49 controls) suggest a strong carcinogenic response. The observation is supported by the fact that the same type of tumors (mouse uterine tumors) was seen with a comparator molecule ethyl bromide, in a more robust carcinogenicity study with 3 doses and a control (Ref. 10).

Ethyl chloride is considered to be a mutagenic carcinogen. Based on the NTP inhalation study the most sensitive species/site is female mouse uterus. Since the number of tumors is high, it is possible to calculate a TD_{50} even though only one dose was tested. The authors of the CPDB (Ref. 8) converted 0 and 15,000 ppm to doses of 0 and 12.4 g/kg and calculated a TD_{50} of 1810 mg/kg/day for mouse uterine tumors.

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 1810 mg/kg/day /50,000 x 50 kg

Lifetime AI = 1,810 µg/day

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Glycidol (CAS# 556-52-5)

Potential for human exposure

Heating of glycerol and sugars causes the formation of glycidol. Glycidol is a metabolite of 3-monochloropropane-1, 2-diol, a chloropropanol found in many foods and food ingredients, including soy sauce and hydrolyzed vegetable protein. Potential daily glycidol exposure in food has been estimated at 20-80 µg/day (Ref. 1).

Mutagenicity/genotoxicity

Glycidol is mutagenic/genotoxic *in vitro* and *in vivo*.

IARC (Ref. 2) and CCRIS (Ref. 3) contain reviews of the mutagenicity/genotoxicity data for glycidol; key conclusions are summarized here.

Glycidol is mutagenic in:
Microbial reverse mutation assay (Ames), *Salmonella* strains TA100, TA1535, TA98, TA97 and TA1537 both with and without rat liver S9 activation and in standard plate and preincubation assays.
Escherichia coli strain WP2*uvrA*/pKM101 in a preincubation assay with and without rat liver S9.

In vivo, glycidol was positive in a mouse micronucleus assay by oral gavage in male and female P16Ink4a/p19Arf haploinsufficient mice.

Carcinogenicity

Glycidol is classified by IARC as Group 2A, or probably carcinogenic in humans (Ref. 2).

In NTP studies (Ref. 4, 5), glycidol was administered by gavage in water to male and female F344/N rats and B6C3F1 mice. Rats received 0, 37.5, or 75 mg/kg and mice received 0, 25, or 50 mg/kg daily, 5 days per week for 2 years. The average daily doses were calculated by multiplying the administered dose by 5/7 to account for the 5 days per week dosing schedule and 103/104 to account for the less-than-lifetime duration of dosing. The resulting average daily doses were 0, 26.5, and 53.1 mg/kg/day in male and female rats, and 0, 17.7, and 35.4 mg/kg/day in male and female mice.

Exposure to glycidol was associated with dose-related increases in the incidences of neoplasms in various tissues in both rats (mammary gland tumors in females), and mice (Harderian gland). Survival of treated rats and mice was markedly reduced compared to controls because of the early induction of neoplastic disease.

The oral gavage study in hamsters was less robust due to small group size, single dose levels and shorter duration. Further oral gavage chronic studies with glycidol were conducted by the NTP in genetically modified mice lacking two tumor suppressor genes (*i.e.*, haploinsufficient p16Ink4a/p19Arf mice) (Ref. 6). Although there was clear evidence of carcinogenic activity in males (based on the occurrence of histiocytic sarcomas and alveolar/bronchiolar adenomas) and some evidence of carcinogenic activity in female mice (based on the occurrence of alveolar/bronchiolar adenomas), these studies are considered less suitable for dose-response

assessment than the two-year bioassays (Ref. 5) for reasons including the short duration, the small number of animals used per treatment group, and limited understanding of how dose-response relationships observed in genetically modified animals correspond with those observed in standard long-term carcinogenicity bioassays (Ref. 7).

Glycidol – Details of carcinogenicity studies

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/sex	TD_{50} (mg/kg/d)
Ref. 5*	50/sex/ group F344/N rats	2 years 5 days/wk Oral gavage	50	**2:** 26.5; 53.8 mg/kg/d	Mammary gland/Female	4.15
Ref. 5	50/sex/ group B6C3F1 mice	2 years 5 days/wk Oral gavage	50	**2:** 17.7; 35.4 mg/kg/d	Harderian gland /Female	32.9
Ref. 8	12-20/ sex/group Syrian Golden Hamsters	60 weeks Twice/wk Gavage	Yes	**1:** M: 15.8 F: 17.9 mg/kg/d	Spleen/Female	56.1^
Ref. 9 (**Cited in Ref. 2)	20 ICR/Ha Swiss mice	520 days 3 times/wk Skin Painting	Yes	**1:** 5%	No Tumors	NA^

Studies listed are in CPDB (Ref. 10) unless otherwise noted.
*Carcinogenicity study selected for AI calculation.
**Not in CPDB.
NA= Not applicable.
^Not a standard carcinogenicity design. Only one dose, intermittent dosing, and small sample size (Ref.7).

Mode of action of carcinogenicity

Glycidol is a mutagenic carcinogen, and the acceptable intake is calculated by linear extrapolation from the TD50.

Regulatory and/or published limits

No regulatory limits have been published, for example by US EPA, WHO, or ATSDR.

Acceptable intake (AI)

Rationale for selection of study for AI calculation

The most suitable carcinogenicity data for human cancer potency assessment come from the two-year oral studies conducted in F344/N rats and B6C3F1 mice by NTP (Ref. 5). The most sensitive organ site was female mammary glands with a TD_{50} of 4.15 mg/kg/day.

Calculation of AI

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 4.15 (mg/kg/day)/50,000 x 50 kg

Lifetime AI = 4 µg/day

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Hydrazine (CAS# 302-01-2)

Potential for human exposure

Hydrazine is used in the synthesis of pharmaceuticals, pesticides and plastic foams (Ref. 1). Hydrazine sulphate has been used in the treatment of tuberculosis, sickle cell anemia and other chronic illnesses (Ref. 2). There is limited information on the natural occurrence of hydrazine and derivatives (Ref. 3). Humans may be exposed to hydrazine from environmental contamination of water, air and soil (Ref. 1); however, the main source of human exposure is in the workplace (Ref. 4). Small amounts of hydrazine have also been reported in tobacco products and cigarette smoke (Ref. 1, 5).

Mutagenicity/genotoxicity

Hydrazine is mutagenic and genotoxic *in vitro* and *in vivo*.

IARC (Ref. 6) has reviewed the mutagenicity of hydrazine. Key observations are summarized here.

Hydrazine was mutagenic in:
Microbial reverse mutation assay (Ames), *Salmonella typhimurium* strains TA 1535, TA 102, TA 98 and TA 100, and in *Escherichia coli* strain WP2 *uvrA*, with and without activation;
In vitro mouse lymphoma L5178Y cells, in *tk* and *hprt* genes.

In vivo, (Ref. 6) hydrazine induced micronuclei but not chromosome aberrations in mouse bone marrow. DNA adducts have been reported in several tissues *in vivo*.

Carcinogenicity

Hydrazine is classified by IARC as Group 2B, or possibly carcinogenic to humans (Ref. 6) and by US EPA as Group B2 or a probable human carcinogen (Ref. 7).

There are seven hydrazine carcinogenicity studies cited in the CPDB (Ref. 8): Three inhalation studies that included 1-year dosing duration, three studies in drinking water and one by oral gavage. Five of the seven hydrazine carcinogenicity studies were deemed positive by the authors of the original reports.

The main target organs for oral carcinogenicity of hydrazine in rodents are the liver and lungs. The most robust oral studies based on group size and dose levels were published in Refs. 9 and 10. The most robust inhalation study with the lowest TD_{50} is in Ref. 11. The most sensitive tumor targets for inhalation carcinogenicity of hydrazine in rodents are sites of initial contact such as the nasal cavity and lungs.

The studies done on hydrazine sulphate in the CPDB (Ref. 8) are not shown here as they included <50 animals per group (and a single dose level in one case), and the calculated TD_{50} values were higher (less potent) than those for the drinking water study of hydrazine (Ref. 9). Given the similarity between the outcomes from the two robust drinking water studies (Ref. 9, 10), the more recent study with the higher tested doses (Ref. 10) was selected for the non-inhalation AI calculation for hydrazine.

Hydrazine – Details of carcinogenicity studies

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/type/sex	TD_{50} (mg/kg/d)
Ref. 9	50/sex/ group Wistar rats	Lifetime Drinking water	50	**3:** M: 0.1; 1.5, 2.5. F: 0.11, 0.57, 2.86 mg/kg/d	Liver/Female	41.6
Ref. 11*	100/sex/ group F344 rats	1 year with 18 mo observation Inhalation	150	**4:** M:1.37, 6.87, 27.5, 137 F: 1.96, 9.81, 39.3, 196 µg/kg/d	Nasal adenamatous polyps/Male	0.194
Ref. 12	50/sex/ group Bor:NMRI, SPF-bred NMRI mice	2 year Drinking water	50	**3:** M: 0.33, 1.67, 8.33. F: 0.4, 2.0, 10.0 mg/kg/d	Negative	NA, negative study
Ref. 11	200 male Golden Syrian hamsters	1 year with 12 mo observation Inhalation	Yes	**3:** 0.02, 0.08, 0.41 mg/kg/d	Nasal adenomatous polyps/Male	4.16
Ref. 11	400 female C57BL/6 Mice	1 year with 15 mo observation Inhalation	Yes	**1:** 0.18 mg/kg/d	Negative	NA
Ref. 13	50/sex/ group Swiss mice	Lifetime Drinking water	Not concurrent	**1:** ~1.7-2 mg/kg/d	Lung/Male	2.20¥
Ref. 14	25 female Swiss mice	40 weeks 5d/wk Gavage	85 Untreated	**1:** ~5 mg/kg/d	Lung/Female	5.67¥¥
Ref. 10**^	50/sex/ F344/DuCrj rats	Lifetime Drinking water	Yes	**3:** M: 0.97, 1.84, 3.86 F:1.28, 2.50, 5.35 mg/kg/d	Liver/Female	38.7
Ref. 10^	50/sex Crj:BDF1 mice	Lifetime Drinking water		**3:** M: 1.44, 2.65, 4.93 F: 3.54,	Liver/Female	52.4

				6.80, 11.45 mg/kg/d		

Studies listed are in CPDB (Ref. 8).
*Carcinogenicity study selected for inhalation AI calculation.
**Carcinogenicity study selected for non-inhalation TD_{50} (see Note 2) and AI calculations.
NA= Not applicable.
¥ Excluded by US EPA (Ref. 7); no concurrent controls. Liver negative.
¥¥ Animal survival affected. Liver negative.
^Not in CPDB

Mode of action of carcinogenicity

Not defined. DNA adducts have been detected *in vivo*, (Ref. 15, 16, 17, 18, 19, 20) although they are reported in tissues that do not develop tumors, so their contribution to tumorigenicity is not known.

Regulatory and/or published limits

The US EPA (Ref. 7) has published an oral slope factor of 3.0 per mg/kg/day and a drinking water unit risk of 8.5×10^{-5} per µg/L. At the 1 in 100,000 risk level, this equates to a concentration of 0.1 µg of hydrazine/L of water or ~0.2 µg/day for a 50 kg/human. This limit is a linearized multistage extrapolation based on the observation of hepatomas in a multi-dose gavage study (Ref. 21) where hydrazine sulfate was administered to mice for 25 weeks followed by observation throughout their lifetime (Ref. 7). Additional studies were identified that were published after the oral slope factor was calculated (Ref. 9, 10, 17, 22). These studies could potentially produce a change in the oral slope factor but it has not yet been re-evaluated by US EPA.

The US EPA (Ref. 7) has also published an inhalation slope factor of 17 per mg/kg/day and an inhalation unit risk of 4.9×10^{-3} per µg/m^3. At the 1 in 100,000 risk level, this equates to an air concentration of 2×10^{-3} µg/m^3 of hydrazine or 0.04 µg/day assuming a person breathes 20 m^3/day. This limit is a linearized multistage extrapolation based on the observation of nasal cavity adenoma or adenocarcinoma in male rats in a multi-dose inhalation study where hydrazine was administered 6 hours/day, 5 days/week for 1 year followed by an 18-month observation period (cited in Ref. 7). Only the US EPA review of this data was accessible; however, the results appear to be very similar to, if not the same as, those of Vernot *et al* (Ref. 11).

Acceptable intake (AI)

Rationale for selection of study for AI calculation

Both oral and inhalation carcinogenicity studies for hydrazine were reviewed to determine if a separate limit is required specific for inhalation carcinogenicity. Given the more potent carcinogenicity specific to the first site-of-contact observed in inhalation studies, it was determined that a separate AI for inhalation exposure was appropriate.

For oral hydrazine, carcinogenicity has been reported in 4 mouse studies and 2 rat studies. The most sensitive effect in the oral studies was based on hepatocellular adenomas and carcinomas of the liver in female rats (Ref. 10).

All of the inhalation carcinogenicity studies that were used by the US EPA in the derivation of the inhalation carcinogenicity limit for hydrazine were taken into consideration when selecting the most robust carcinogenicity study for the derivation of an AI for inhaled pharmaceuticals. The critical study by MacEwen *et al* used by US EPA (Ref. 7) was proprietary but is likely the same one described in Vernot *et al* (Ref. 11). Given that the TTC was derived *via* linear extrapolation from TD_{50} values for hundreds of carcinogens, that same approach was used in the derivation of a compound-specific AI for hydrazine. The methodology used by the US EPA and the method used here are both highly conservative in nature. However, given that the methodologies do differ, it is reasonable to expect some slight differences. The AI was calculated based on the TD_{50} derived from a study in which male and female rats were administered hydrazine *via* inhalation for one year with an 18-month observation period (Ref. 11). While a 1-year study is not a standard design for carcinogenicity, a positive response was observed demonstrating that the window for carcinogenicity was not missed. The most sensitive target tissue was the male nasal region, with a TD_{50} value of 0.194 mg/kg/day, after being adjusted, as standard practice, to account for 1 vs 2 years of exposure.

Calculation of AI

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 38.7 (mg/kg/day)/50,000 x 50 kg

Lifetime AI = 39 µg/day

Calculation of inhalation AI

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 0.194 (mg/kg/day)/50,000 x 50 kg

Lifetime inhalation AI = 0.2 µg/day

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Hydrogen Peroxide (CAS# 7722-84-1)

Potential for human exposure

Hydrogen peroxide can be present in green tea and instant coffee, in fresh fruits and vegetables and naturally produced in the body (Ref. 1). It is estimated up to 6.8 g is produced endogenously per day (Ref. 2). Other common sources of exposure are from disinfectants, some topical cream acne products, and oral care products which can contain up to 4% hydrogen peroxide (Ref. 2).

Mutagenicity/genotoxicity

Hydrogen peroxide is mutagenic and genotoxic *in vitro* but not *in vivo*.

IARC (Ref. 3) and European Commission Joint Research Centre (Ref. 4) reviewed the mutagenicity data for hydrogen peroxide, and key observations are summarized here.

Hydrogen peroxide is mutagenic in:
Salmonella typhimurium strains TA96, TA97, SB1106p, SB1106, and SB1111 and *Escherichia coli* WP2 in the absence of exogenous metabolic activation;
L5178Y mouse lymphoma cell sublines at the *hprt* locus;
Chinese hamster V79 cells at the *hprt* locus, in only one of six studies.

In vivo, micronuclei were not induced after administration of hydrogen peroxide to mice intraperitoneally at up to 1,000 mg/kg, or to catalase-deficient C57BL/6NCr1BR mice in drinking water at 200, 1,000, 3,000, and 6,000 ppm for two weeks.

Carcinogenicity

Hydrogen peroxide is classified by IARC as Group 3, not classifiable as to its carcinogenicity to humans (Ref. 3).

There is only one carcinogenicity report (Ref. 5) cited in the CPDB (Ref. 6), in which mice were treated with hydrogen peroxide in drinking water at 0.1 or 0.4% for approximately 2 years. The study included two treatment groups and about 50 animals per dose group. Statistically significant increases in tumors of the duodenum ($p<0.005$) were observed in both dose groups in the mouse carcinogenicity study (Ref. 5) although only the duodenal tumors at the high dose in females are noted as significant in the CPDB (Ref. 6). Thus, 0.1% hydrogen peroxide administered in drinking water was defined as the Lowest Observed Adverse Effect Level (LOAEL), equivalent to an average daily dose-rate per kg body weight per day of 167 mg/kg/day.

Studies of 6-month duration or longer are summarised in the following table (adapted from Ref. 2); they are limited in the numbers of animals and used a single dose level. Most studies did not meet the criteria for inclusion with a TD_{50} calculation in the CPDB. DeSesso *et al* (Ref. 2) noted that, out of 14 carcinogenicity studies (2 subcutaneous studies in mice, 2 dermal studies in mice, 6 drinking water studies [2 in rats and 4 in mice], 1 oral intubation study in hamsters, and 3 buccal pouch studies), only 3 mouse drinking water studies (Ref. 5, 8, 9) demonstrated increases in tumors (of the proximal duodenum) with hydrogen peroxide. These mouse studies were thoroughly evaluated by the Cancer Assessment Committee (CAC)

of the US FDA (Ref. 10). The conclusion was that the studies did not provide sufficient evidence that hydrogen peroxide is a carcinogen (Ref. 10).

In Europe, the Scientific Committee on Consumer Products reviewed the available data for hydrogen peroxide and concluded that hydrogen peroxide did not meet the definition of a mutagen (Ref.11) They also stated that the weak potential for local carcinogenic effects has an unclear mode of action, but a genotoxic mechanism could not be excluded (Ref. 11). In contrast, DeSesso *et al* (Ref. 2) suggested that dilute hydrogen peroxide would decompose before reaching the target site (duodenum) and that the hyperplastic lesions seen were due to irritation from food pellets accompanying a decrease in water consumption, which is often noted with exposure to hydrogen peroxide in drinking water. The lack of a direct effect is supported by the lack of tumors in tissues directly exposed *via* drinking water (mouth, oesophagus and stomach), and the fact that in studies up to 6 months in the hamster (Ref. 14), in which hydrogen peroxide was administered by gastric intubation (water intake was not affected), the stomach and duodenal epithelia appeared normal; this was the basis for the US FDA conclusion above (Ref. 10).

Hydrogen Peroxide – Details of oral carcinogenicity studies

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Notes
Ref. 5*	48-51/sex/ group C57BL/6J mice	100 weeks Drinking water	Yes	**2:** 0.1; 0.4% M: 167; 667 F: 200; 800 mg/kg/d	TD_{50} 7.54 g/kg/d for female duodenal carcinoma
Ref. 7	29 mice C57BL/6J total male & female (additional groups sampled at intervals from 7 to 630 days of treatment; or 10 – 30 days after cessation of treatment at 140 days)	700 days Drinking water	No	**1:** 0.4%	No tumors reported. Time-dependent induction of erosions and nodules in stomach and nodules and plaques in duodenum. After a recovery period following 140 days of H_2O_2 treatment, by 10 to 30 days without treatment there were fewer mice with lesions.
Ref. 8	18 C3H/HeN mice total male & female	6 mo Drinking water	No	**1:** 0.4%	2 mice with duodenal tumors (11.1%)
Ref. 8	22 B6C3F1 mice total male & female	6 mo Drinking water	No	**1:** 0.4%	7 mice with duodenal tumors (31.8%)
Ref. 8	21 C57BL/6N ¢ mice total male &	7 mo Drinking water	No	**1:** 0.4%	21 mice with duodenal tumors (100%)

	female				
Ref. 8	24 C3HCb/s ¢ mice total male & female	6 mo Drinking water	No	0.4% only	22 mice with duodenal tumors (91.7%)
Ref. 9	21 female C3H/HeN mice	6 mo Drinking water	11	**1:** 0.4%	2 mice with duodenal tumors (9.5%). None in controls
Ref. 9	22 female B6C3F1 Mice	6 mo Drinking water	12	**1:** 0.4%	7 mice with duodenal tumors (31.8%) None in controls
Ref. 9	24 female C3HCb/s ¢ mice	6 mo Drinking water	28	**1:** 0.4%	22 mice with duodenal tumors (91.7%). None in controls
Ref. 12	3 male rats	21 weeks Drinking water	3	**1:** 1.5%	No tumorigenic effect observed
Ref. 13	Male and female rats (50/sex/group)	2 years Drinking water	Yes	**2:** 0.3% 0.6%	No tumorigenic effect observed
Ref. 14	Hamsters, sex not reported (20/group)	15 weeks and 6 mo Oral gavage (5 d/wk)	Yes	**1:** 70 mg/kg/d	No tumorigenic effect observed

*Carcinogenicity study selected for PDE calculation; in CPDB (Ref. 6).
All other studies are not in the CPDB but are summarized in Ref. 2
¢ Catalase deficient

Mode of action for carcinogenicity

Hydrogen peroxide is one of the reactive oxygen species (ROS) that is formed as part of normal cellular metabolism (Ref. 4). The toxicity of hydrogen peroxide is attributed to the production of ROS and subsequent oxidative damage resulting in cytotoxicity, DNA strand breaks and genotoxicity (Ref. 15). Due to the inevitable endogenous production of ROS, the body has evolved defense mechanisms to limit their levels, involving catalase, superoxide dismutases and glutathione peroxidase.

Oxidative stress occurs when the body's natural antioxidant defense mechanisms are exceeded, causing damage to macromolecules such as DNA, proteins and lipids. ROS also inactivate antioxidant enzymes, further enhancing their damaging effects (Ref. 16). During mitochondrial respiration, oxygen undergoes single electron transfer, generating the superoxide anion radical. This molecule shows limited reactivity but is converted to hydrogen peroxide by the enzyme superoxide dismutase. Hydrogen peroxide is then reduced to water and oxygen by catalase and glutathione peroxidase (Ref. 17). However, in the presence of transition metals, such as iron and copper, hydrogen peroxide is reduced further to extremely reactive hydroxyl radicals. They are so reactive they do not diffuse more than one or two molecular diameters before reacting with a cellular component (Ref. 16). Therefore, they must be generated immediately adjacent to DNA to oxidize it. Antioxidants provide a source

of electrons that reduce hydroxyl radicals back to water, thereby quenching their reactivity. Clearly, antioxidants and other cellular defenses that protect against oxidative damage are limited within an *in vitro* test system. Consequently, following treatment with hydrogen peroxide these protective mechanisms are readily overwhelmed inducing cytotoxicity and genotoxicity in bacterial and mammalian cell lines. Diminution of the *in vitro* response has been demonstrated by introducing elements of the protective mechanisms operating in the body; for example, introducing hydrogen peroxide degrading enzymes, such as catalase or adjusting the level of transition metals (Ref. 11). Unsurprisingly, *in vivo*, where the cellular defense mechanisms are intact, hydrogen peroxide is not genotoxic following short-term exposure. This suggests that a threshold exists below which the cellular defense mechanisms can regulate ROS maintaining homeostasis.

Based on the comprehensive European Commission (EC, Ref. 4) risk assessment, the weight of evidence suggests hydrogen peroxide is mutagenic *in vitro* when protective mechanisms are overwhelmed. However, it is not genotoxic in standard assays *in vivo*. Its mode of action has a non-linear, threshold effect.

Regulatory and/or published limits

Annex III of the European Cosmetic Regulation (Ref. 18) provided acceptable levels of hydrogen peroxide in oral hygiene and tooth whitening products. For oral products sold over the counter, including mouth rinse, toothpaste and tooth whitening or bleaching products, the maximum concentrations of hydrogen peroxide allowed (present or released) is 0.1%. Higher levels up to 6% are also permitted providing products are prescribed by dental practitioners to persons over 18 years old. The EC SCCP (Ref. 11) estimated that 3 g of mouthwash or 0.48 g of toothpaste could be ingested per day. With 0.1% hydrogen peroxide in the product, the amount of hydrogen peroxide potentially ingested would be 3 mg from mouthwash or 0.48 mg from toothpaste. These values may overestimate ingestion as it is likely that most of the hydrogen peroxide is decomposed during use of oral care products and is not ingested (Ref. 4).

US FDA - hydrogen peroxide is Generally Recognized As Safe (GRAS) up to 3% for long-term over the counter use as an anti-gingivitis/anti-plaque agent (Ref. 19).

Permissible daily exposure (PDE)

Hydrogen peroxide is genotoxic *via* a mode of action with a threshold (i.e., oxidative stress) and is endogenously produced in the body at high levels that exceed the levels encountered in oral care and other personal care products. Therefore it was not considered appropriate to derive a PDE based on carcinogenicity data. Even an intake 1% of the estimated endogenous production of 6.8 g/day, that is, 68 mg/day (or 68,000 µg/day) would not significantly add to background exposure, but would usually exceed limits based on quality, in a pharmaceutical. The ICH M7 guideline notes that when calculating acceptable intakes from compound-specific risk assessments, an upper limit would be determined by a quality limit of 0.5%, or, for example, 500 µg in a drug with a maximum daily dose of 100 mg.

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Methyl Chloride (Chloromethane, CAS# 74-87-3)

Potential for human exposure

Low levels of methyl chloride occur in the environment, since thousands of tons of methyl chloride are produced naturally every day, e.g., by marine phytoplankton, by microbial fermentation, and from biomass fires (burning in grasslands and forest fires) and volcanoes, greatly exceeding release from human activities.

WHO (Ref. 1) reports that the methyl chloride concentration in the air in rural sites is in general below 2.1 µg/m^3 (1.0 ppb) while in urban cities it is equal to 0.27 to 35 µg/m^3 (0.13-17 ppb), corresponding to approximately 20-700 µg daily intake (human respiratory volume of 20 m^3 per day). A wide range of concentrations is reported in rivers, ocean water, ground water and drinking water, with the maximum drinking water level reported at 44 µg/L in a well sample (Ref. 1).

Mutagenicity/Genotoxicity

Methyl chloride is mutagenic and genotoxic *in vitro* but equivocal *in vivo*. WHO (Ref. 1) and US EPA (Ref. 2) reviewed the mutagenicity data for methyl chloride; key observations are summarized here.

Methyl chloride is mutagenic in:
Microbial reverse mutation assay (Ames), *Salmonella typhimurium* TA100, TA1535 and in *Escherichia coli* WP2*uvrA* both in the presence and absence of metabolic activation;
TK6 human lymphoblasts.

In vivo, WHO (Ref. 1) concluded that "though data from standard *in vivo* genotoxicity studies are not available, methyl chloride might be considered a very weak mutagen *in vivo* based on some evidence of DNA–protein crosslinking at higher doses".

Carcinogenicity

Methyl chloride is classified by IARC as Group 3: "Inadequate evidence for the carcinogenicity to humans" (Ref. 3), and by US EPA as a Category D compound not classifiable as to human carcinogenicity (Ref. 2).

In animals, the only evidence of carcinogenicity comes from a single 2-year bioassay that used the inhalation route of administration in rats and mice (Ref. 4). A statistically significant increased incidence of renal benign and malignant tumors was observed only in male B6C3F1 mice at the high concentration (1,000 ppm). Although not of statistical significance, cortical adenoma was also seen at 464 mg/m^3 (225 ppm), and development of renal cortical microcysts in mice was seen in the 103 mg/m^3 (50 ppm) dose group and to some extent in the 464 mg/m^3 (225 ppm) group (Ref. 4). However, no concentration–response relationship could be established. Renal cortical tubuloepithelial hyperplasia and karyomegaly were also confined to the 1,000-ppm group of male mice. Neoplasias were not found at lower concentrations or at any other site in the male mouse, or at any site or concentration in female mice or F-344 rats of either sex. Renal adenocarcinomas have been shown to occur only in male mice at a level of exposure unlikely to be encountered by people.

These renal tumors of the male mouse are not likely to be relevant to humans. Methyl chloride is metabolized by glutathione conjugation and to a lesser extent by p450 oxidation (Ref. 1, 2). Renal tumors in male mouse are thought to be related to the production of formaldehyde during methyl chloride metabolism. The cytochrome P-450 (CYP) isozyme believed to be responsible, CYP2E1, is present in male mouse kidney and is androgen-dependent; female mice had CYP2E1 levels only 20-25% of those in males. Generation of formaldehyde has been demonstrated in renal microsomes of male CD-1 mice that exceed that of naive (androgen-untreated) female mice, whereas kidney microsomes from the rat did not generate formaldehyde. Additionally, species-specific metabolic differences in how the kidney processes methyl chloride strongly suggest that renal mouse neoplasms *via* P-450 oxidation are not biologically relevant to humans given that human kidney lacks the key enzyme (CYP2E1) known to convert methyl chloride to toxic intermediates having carcinogenic potential. In the rat, renal activity of CYP2E1 was very low. No CYP2E1 activity was detected in human kidney microsomal samples (Ref. 2), nor was it detected in freshly isolated proximal tubular cells from human kidney. CYP4A11 was detected in human kidney, but its ability to metabolize methyl chloride is unknown. In addition to CYP4A11, the only other P-450 enzymes found at significant levels in human renal microsomes are CYP4F2 and CYP3A. Moreover no commonly known environmental chemicals appear to be metabolized by the CYP4A family. The lack of detectable CYP2E1 protein in human kidney (in contrast to mice, which have high levels) suggests that the metabolism of methyl chloride by P450 (presumably leading to elevated formaldehyde concentrations) that is likely responsible for the induction of male mouse kidney tumors are not likely relevant to humans.

However, as highlighted by the US EPA (Ref. 2) and WHO (Ref. 1), the role of hepatic (and/or kidney) metabolism (leading to potential genotoxic metabolites) *via* the predominant glutathione (GSH)-dependent pathway (metabolism of methyl chloride to formate in liver is GSH-dependent, *via* the GSH-requiring formaldehyde dehydrogenase that oxidizes formaldehyde to formate) or even by P450 isozymes other than CYP2E1 in this regard cannot be discounted. Nonetheless, production of formaldehyde *via* low doses of methyl chloride would be negligible compared with the basal formation of formaldehyde in the body (i.e., 878–1310 mg/kg/day; Ref. 5). In addition, based on the limitations of human relevance, US EPA classified methyl chloride as a group D compound, that is “Not Classifiable as to Human Carcinogenicity".

Methyl Chloride – Details of carcinogenicity studies (only inhalation studies available)

Study	Animals/ dose group	Duration/ Exposure	Controls	Doses	Most sensitive tumor site/sex	TD_{50} (mg/kg/d)
Ref. 4 (summarized in Ref. 1 and Ref. 2)*	120/sex/ group B6C3F1 mice	24 mo 6h/d, 5d/wk Inhalation	Yes	**3:** 103; 464; 2064 mg/m^3 (50; 225; 1000 ppm)	Kidney tumors in males only. No finding in females.	1,360.7**
Ref. 4 (summarized in Ref. 1 and Ref. 2)	120/sex/ group Fisher 344 rats	24 mo 6h/d, 5d/wk Inhalation	Yes	**3:** 103; 464; 2064 mg/m^3 (50; 225; 1000 ppm)	No findings in males and females	NA

Note: Studies not listed in CPDB.
*Carcinogenicity study selected for AI calculation.
**TD_{50} calculated based on carcinogenicity data (see Note 3).
NA = Not applicable

Regulatory and/or published Limits

WHO (Ref. 1) developed a guideline value for the general population of 0.018 mg/m^3 and US EPA (Ref. 2) developed a reference concentration of 0.09 mg/m^3. Both were based on the potential for adverse CNS effects following inhaled methyl chloride.

Acceptable intake (AI)

While the data indicate the tumors observed in male mice are likely not relevant to humans, an AI was developed because of the uncertainties in data.

Lifetime AI = TD_{50}/50,000 x 50 kg

Lifetime AI = 1,360.7 mg/kg/day /50,000 x 50 kg

Lifetime AI = 1,361 µg/day

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Note 1

The calculated TD_{50} for 1-chloro-4-nitrobenzene is illustrated below since it was not listed in the CPDB. 1-Chloro-4-nitrobenzene calculations were based on the most sensitive tumor type: female rat pheochromocytoma (Ref. 1). The doses and incidences are listed below.

ppm	Dose (mg/kg/day)	Number of Positive Animals	Total Number of Animals
0	0	3	50
50	1.9	6	50
225	9.8	4	50
1000	53.8	16	50

\

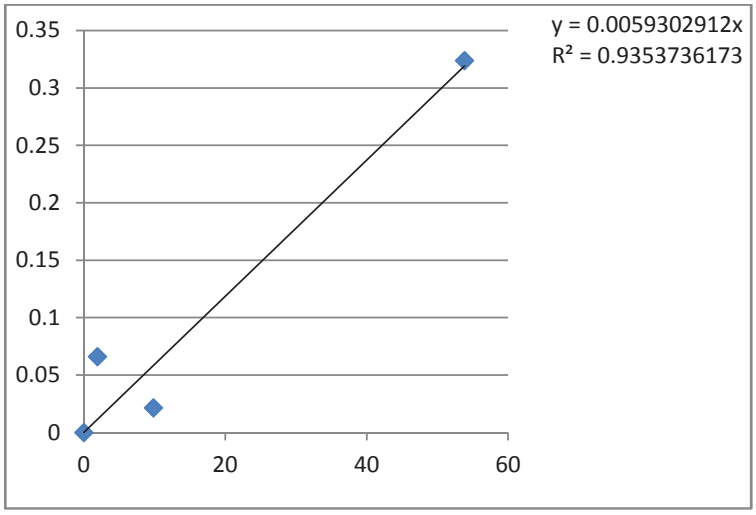
The TD_{50} is calculated from crude summary data of tumor incidence over background with the following equation (Ref. 2, 3):

$$\frac{P - P_0}{1 - P_0} = 1 - \exp(-\beta \cdot D)$$

Where P is the proportion of animals with the specified tumor type observed at a certain dose (D in the equation) and P_0 is the proportion of animals with the specified tumor type for the control. Converting β and D into a simple linear equation results in the following:

$$\ln\left(-[\frac{P - P_0}{1 - P_0} - 1]\right) = \beta \cdot D$$

Plotting the results and using the slope to represent β results in the following graph for the dose-response and β = 0.0059302912.



The TD_{50} can then be calculated as follows.

$$0.5 = 1 - \exp(-\beta \cdot TD_{50})$$

Solving for TD_{50} results in in the following equation.

$$TD_{50} = \frac{0.693}{\beta}$$

Therefore, the TD_{50} = 0.693 / 0.0059302912 or 116.9 mg/kg/day.

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Note 2

The calculated TD_{50} for hydrazine is illustrated below since it was not listed in the CPDB. Hydrazine calculations were based on the most sensitive tumor type: female rats, hepatocellular adenoma and/or carcinoma (Ref. 1). The doses and incidences are listed below

ppm	Dose (mg/kg/day)	Number of Positive Animals	Total Number of Animals
0	0	1	50
20	1.28	0	50
40	2.50	3	50
80	5.35	6	50

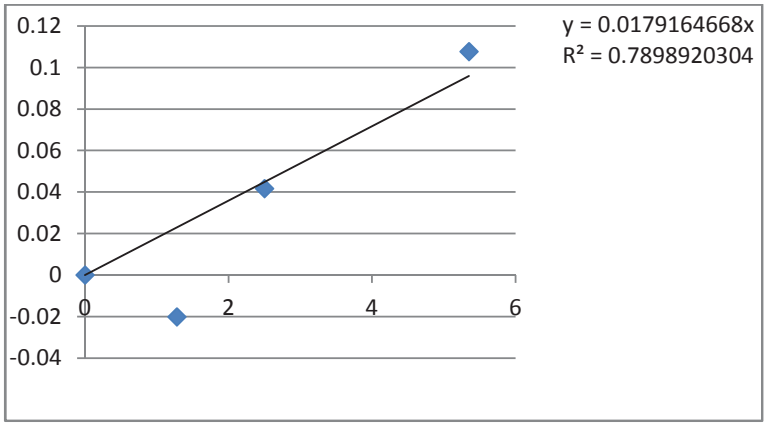
The TD_{50} is calculated from crude summary data of tumor incidence over background with the following equation (Ref. 2, 3):

$$\frac{P - P_0}{1 - P_0} = 1 - \exp(-\beta \cdot D)$$

Where P is the proportion of animals with the specified tumor type observed at a certain dose (D in the equation) and P_0 is the proportion of animals with the specified tumor type for the control. Converting β and D into a simple linear equation results in the following:

$$\ln\left(-\left[\frac{P - P_0}{1 - P_0} - 1\right]\right) = \beta \cdot D$$

Plotting the results and using the slope to represent β results in the following graph for the dose-response and β = 0.0179164668.



The TD_{50} can then be calculated as follows.

$$0.5 = 1 - \exp(-\beta \cdot TD_{50})$$

Solving for TD_{50} results in in the following equation.

$$TD_{50} = \frac{0.693}{\beta}$$

Therefore, the TD_{50} = 0.693 / 0.0179164668 or 38.7 mg/kg/day.

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Note 3

The calculated TD_{50} for methyl chloride is illustrated below since it was not listed in the CPDB. Since the methyl chloride study (Ref. 1, 2) is based on inhalation, the inhaled ppm concentrations need to be converted to dose.

ppm	Dose (mg/kg/day)[1]	Number of Positive Animals	Total Number of Animals
0	0	0	67
50	28	0	61
225	127	2	57
1000	566	22	86

1. ppm to mg/kg/day conversion – X ppm x 50.5 g/mol (mol weight)/24.45 x 0.043 (breathing volume) x 6/24 hours x 5/7 days / 0.028 kg (mouse weight) = dose mg/kg/day

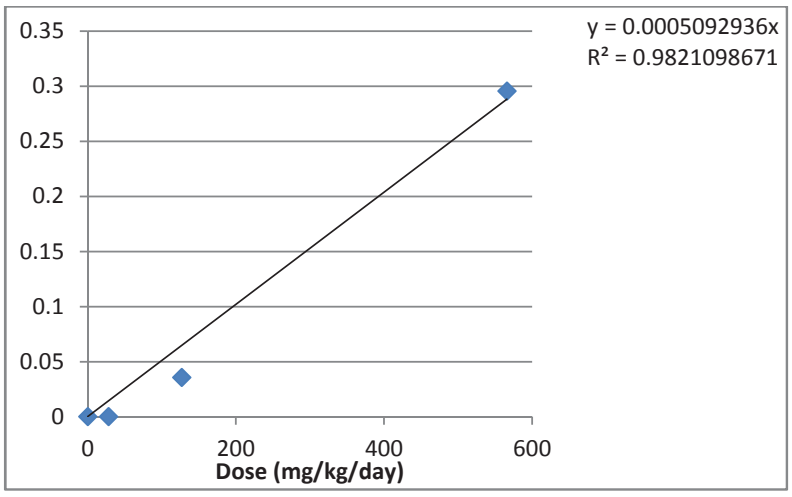
The TD_{50} is calculated from crude summary data of tumor incidence over background with the following equation (Ref. 3, 4):

$$\frac{P - P_0}{1 - P_0} = 1 - \exp(-\beta \cdot D)$$

Where P is the proportion of animals with the specified tumor type observed at a certain dose (D in the equation) and P_0 is the proportion of animals with the specified tumor type for the control. Converting β and D into a simple linear equation results in the following:

$$\ln\left(-[\frac{P - P_0}{1 - P_0} - 1]\right) = \beta \cdot D$$

Plotting the results and using the slope to represent β results in the following graph for the dose-response and β = 0.0005092936.



The TD_{50} can then be calculated as follows.

$$0.5 = 1 - \exp(-\beta \cdot TD_{50})$$

Solving for TD_{50} results in in the following equation.

$$TD_{50} = \frac{0.693}{\beta}$$

Therefore, the TD_{50} = 0.693 / 0.0005092936 or 1360.7 mg/kg/day.

References

1. CIIT. Final report on a chronic inhalation toxicology study in rats and mice exposed to methyl chloride. Report prepared by Battelle Columbus Laboratories for the CIIT. 1981 EPA/OTS Doc #878212061, NTIS/OTS0205952.

2. US EPA. Toxicological review of methyl chloride. (CAS No. 74-87-3). In Support of Summary Information on the IRIS. EPA/635/R01/003. 2001.

3. Gaylor DW, Gold LS. Quick estimate of the regulatory virtually safe dose based on the maximum tolerated dose for rodent bioassays. Regul Toxicol Pharmacol.1995; 22:57-63.

4. Sawyer C, Peto R, Bernstein L, Pike MC. Calculation of carcinogenic potency from long-term animal carcinogenesis experiments. Biometrics 1984; 40: 27-40.